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HEMOPHILIA

*The Wesley M. Carpenter Lecture**

WILLIAM H. HOWELL

THE early history of hemophilia is given by Nasse,[1] Bulloch and Fildes,[2] Schloessmann[3] and others.[4] The disease was first definitely described by Dr. John C. Otto[5] of Philadelphia in 1803. His cases and the subsequent fuller collections made by Nasse gave rise to an abundant clinical literature which in time has been followed by extensive experimental investigations starting with the important researches of Sahli[6] in 1905.

The literature up to 1911 has been reviewed critically in the very valuable memoir of Bulloch and Fildes[2] published in that year by the Francis Galton Laboratory for National Eugenics as Parts 5 and 6 of their collection of family pedigrees "illustrating the inheritance in man of mental and physical characters."

These authors sum up their conclusions in the definition that "hemophilia is an inherited tendency in males to bleed." The definition emphasizes what they considered to be the three cardinal characteristics of the disease, namely, the tendency to bleed, the fact that this tendency is inherited and that it is limited to males. It had been further demonstrated

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at this time that the disease is inherited through the female in accordance with the law of Nasse,[1] which, in substance, states that the disease is limited to the male but is transmitted from the male through an unaffected daughter to a grandson. Since 1911 two further conclusions in regard to the disease have met with general acceptance. First, that hemophilia, in the Mendelian nomenclature, behaves as a sex-linked recessive factor; second, that its outstanding diagnostic characteristic is an abnormally prolonged coagulation time of the blood.

The statement that hemophilia is transmitted as a sex-linked recessive means that the factors or genes responsible for its development are contained in the X chromosome of the reproductive cells. This conception enables us to understand the law of Nasse and to predict under what conditions of mating hemophilia may appear in the offspring. The formulae by means of which such predictions may be made are given in the accompanying diagrams. It is assumed that the ovum contains a pair of X-chromosomes (XX), while the spermatozoon has only a single X-chromosome, as a member of the XY pair, the Y-chromosome apparently not carrying sex genes. At the reduction division which takes place during the maturation of the germ-cell each matured ovum will contain a single X-chromosome, but the spermatozoa fall into two groups, one containing an X-chromosome and one without, having instead a Y-chromosome. In the subsequent combination of these sex cells that takes place in fertilization two groupings of the sex-chromosomes are possible. One of these, an XX pair, leads to the development of a female, the other, an XY pair (or a single X-chromosome) gives rise to a male. If in such a mating the male happens to be a hemophiliac then by theory the X-chromosome in his spermatozoa contains the factor for the disease, and in his marriage to a normal female the defect will be transmitted to some of his children. Theoretically, the combination of sex cells will give rise to fertilized ova containing a single normal X-chromosome, which will develop into males, and others, containing a pair of X-chromosomes, one of which carries the hemophilic factor, which will develop into females. Consequently all of his sons will be normal in the sense that they are free of the hemophilic factor, and they will not be capable of transmitting the disease. All the daughters, on the contrary, will have the hemophilic factor. They will not themselves be bleeders since the ovum contains one normal X-chromosome which is dominant in development, but they will be carriers or conductors of the defect and capable of transmitting it to

HEMOPHILIC MALE + NORMAL FEMALE

1. X X CONDUCTOR DAUGHTER
2. X X " "
3. X Y NORMAL SON
4. X Y " "

FIGURE 1

NORMAL MALE + CONDUCTOR FEMALE

1. XX NORMAL DAUGHTER
2. X X CONDUCTOR DAUGHTER
3. XY NORMAL SON
4. XY HEMOPHILIC SON

FIGURE 2

HEMOPHILIC MALE + CONDUCTOR FEMALE

1. X X CONDUCTOR DAUGHTER
2. XX HEMOPHILIC DAUGHTER
3. X Y NORMAL SON
4. XY HEMOPHILIC SON

FIGURE 3

some of their sons, as is indicated in Figure 2, which indicates what may be expected from the marriage of a conductor female to a normal male. Some of her sons may be hemophiliacs and some of her daughters may be conductors, theoretically a fifty-fifty chance in each case.

These predictions fall in with the facts actually known in regard to the transmission of the disease. A hemophilic male married to a normal female never has hemophilic sons, nor, so far as is known, do any of his descendants through his sons suffer from the disease. On the other hand, his daughters' sons frequently exhibit hemophilia and his daughters' daughters may continue to act as conductors and thus pass on the defect in latent form through many generations.

If it should happen that a hemophilic male married a conductor female, then, according to theory he might have a hemophilic daughter as well as a hemophilic son. As Figure 3 indicates, there would be an even chance for the daughters to be conductors or active hemophiliacs.

As a matter of fact no authentic case of a female bleeder is known. Many such cases have been described, but Bulloch and Fildes[2] who reviewed the literature up to 1911, and Bucura[7] and Schloessmann[3] who have discussed the point for the later literature, agree that the evidence is unsatisfactory. What has been described as hemophilia was more probably purpura or some hemorrhagic condition other than hemophilia. In no case were the data given entirely convincing. It seems quite probable that bleeder-conductor marriages may have occurred occasionally among the known hemophilic families inhabiting a certain district, but Schloessmann was able to find only one clear case among the pedigrees on record. In that case the progeny was too small, one son and one daughter, to constitute a crucial test of the theory. The daughter was not hemophilic—whether she was a conductor is not known since there is no record of her marriage. In view of the interest the question has aroused it seems likely that if any clear-cut case of a female hemophiliac has occurred it would have been made a matter of record. That no case has actually been observed may be due solely to the accident that bleeder-conductor marriages are rare, and if they occur the daughters, if any, have an equal chance of being conductors rather than bleeders. Still the apparently complete absence of such cases has aroused comment and has led to the suggestion that there may be some fundamental biological reason operating against their development. Bauer[8] has suggested, for example, that the hemophilic genes may act as a lethal factor (or be associated with a

lethal factor) and that a double dose of this factor, such as would occur in the ovum destined to produce a female bleeder, would make development impossible.

Although hemophilia is so clearly an hereditary disease there is evidence that sporadic cases occur in which inheritance cannot be demonstrated. Bulloch and Fildes[2] discuss the matter briefly and express the opinion that fewer such cases would be reported "if the authors had had sufficient time, interest or perseverance to investigate beneath what might be immediately apparent upon the surface." They refer however specifically to one pedigree, that published by Gettings, which seems to establish the point. In a family of eleven children, six boys and five girls, five of the boys were hemophiliacs. Examination of the medical histories of the direct and collateral ancestry through three generations disclosed no case of hemophilia. Davidson and McQuarrie[9] cite a similar instance in which an only child, a son, was hemophilic. None of the members of either family traced back through three or four generations gave any evidence of the disease, although on the maternal side there were ten males who might have been subject to hemophilia according to Nasse's law. Schloessmann[3] discusses the matter at considerable length. He discounts many of the reported cases on the ground that sufficient inquiry was not made into the family histories, but he admits that cases of genuine hemophilia may occur apparently *de novo*. In such cases the symptoms are essentially identical with those of hereditary hemophilia. They seem to be exactly the same disease. It is difficult to believe that in such cases the disease has developed *de novo* in the individual as an acquired characteristic that will disappear at his death, especially as in some of the recorded cases, that of Gettings, for example, it has appeared in two or more males of the same family. One inclines rather to the alternative view that it is a true hereditary condition which has arisen *de novo* by a mutation of the sex-cells of the mother. From this point of view sporadic cases mark the beginning of a new hemophilic strain. Such presumably was the origin of the known hemophilic families that are on record. However, so far as I can ascertain, actual histories, that might support this theory, are lacking in regard to the descendants of known sporadic cases.

In regard to the second point, noted above, namely the prolongation of the clotting time of the blood, it was known to the early observers that hemophilic blood clots slowly, but in their observations emphasis was laid upon the obstinate character of the bleeding. Sahli[6] was the first to

study systematically variations in the clotting time and since his investigations experimental work has been concentrated chiefly upon the solution of this problem. It is true that many investigators have expressed their belief that the prolonged clotting time is not in itself sufficient to account for the long continued hemorrhages exhibited by the hemophiliac. It has been suggested that there must be also some structural or functional abnormality in the walls of the blood vessels or in the tissues generally to account for the prolonged bleeding, especially as it has been asserted by several good observers that bleeding, traumatic or after operation, may continue when the coagulation time is normal.

The few cases in the literature in which a normal coagulation time has been recorded for hemophilic blood are open to question on the ground of inadequate technique. For while the procedure of determining clotting time is simple, the process of clotting is apparently subject to large variations from very small changes in conditions. It is now generally recognized that methods in which the specimen of blood is obtained by pricking or stabbing the skin are unreliable on account of the possibility of admixture of more or less of thromboplastic substance from the tissues. Since the older observers used this method their results can not be accepted without confirmation. The usual practice at present is to remove the sample of blood by venipuncture with a syringe, but with this method also duplicate samples may show considerable variation in time of clotting. Failure to enter the vein promptly at the first insertion may result in the accumulation of some tissue juice in the needle which will affect the time of clotting. Some of the contradictory results found in the literature are probably to be explained by errors of this kind in the technique.

With regard to pathological changes other than the lengthening of the time of coagulation it may be said that none has been definitely demonstrated. Claims of various kinds have been made with regard to differences in vascular structure or function, or in thromboplastic content of the tissues, or in the composition of the blood, but they have either failed of confirmation at the hands of other observers, or they seem to be lacking in significance. It is possible that some of the minor differences that have been noted, such as the higher chloride content of hemophilic blood, may prove later to be of importance, but at present the one pathological condition that is definitely established and that is directly connected with the hemorrhages of hemophilia is the prolonged clotting time of the blood. Those who have had the widest experience with

hemophilic patients believe that the severity of the disease is in general proportional to the delay in coagulation. On the other hand it is known that remedial measures, such as blood transfusions, which serve to control the hemorrhages tend to restore the coagulation time to or toward the normal. It is believed therefore that a definite prolongation of the clotting time is diagnostic of hemophilia and serves to differentiate it from other hemorrhagic conditions, especially from thrombocytopenic purpura with which it has often been confused. Ordinarily at least the distinction between these two conditions is easily made. In hemophilia the clotting time is prolonged but the platelet count is normal. In thrombocytopenic purpura the coagulation time is normal while the platelet count is much reduced. There is also a difference in the character and location of the hemorrhages. It is interesting to note, however, that several authors have reported cases of a seemingly intermediate character which indicate the possibility of some kind of a connection between the two conditions. Pickering[10] describes a case in which during the period of observation, about two years, the patient seemed to oscillate periodically between hemophilia and purpura—at one time a coagulation time of forty to sixty minutes and a platelet count of 340,000, and a year later a normal clotting time of eleven to twelve minutes but a platelet count of 84,000. Minot and Lee[11] report a similar experience. Some of their cases they say, "at one time may show a normal platelet count, but a marked delay of the coagulation time, while at other times the coagulation time may be less delayed, while the blood-platelets are only few in number." In one such case in my own experience the hemorrhages were of the hemophilic type, affecting the joints, and the coagulation time was somewhat prolonged, twenty-six minutes, but at the same time the platelet count was quite low, 64,000. The patient gave no family history of hemophilia. Unfortunately none of these cases has been followed over an adequate period of time and with reference to their family histories. Their significance remains uncertain but they do suggest caution in making a positive distinction between the two conditions. It may be said that a prolonged coagulation time is found in all cases of undoubted hemophilia, but, used as a diagnostic test, it should be supported by collateral evidence based upon the character of the hemorrhages or the family history.

Data are lacking in regard to the amount of delay in coagulation which may be considered as indicative of a hemophilic state. Indeed we have no satisfactory information in regard to the variations in this respect

that may be exhibited by the blood of normal persons. We can not well compare figures published by different authors owing to variations in the methods used. No observer, so far as I know, has made extensive observations of this kind under varying conditions of health and disease using acceptable and consistent technique throughout.

Schloessmann[3] gives data for twenty of his hemophilic patients. The samples of blood were obtained by venipuncture and the coagulation time was determined by Bürker's method for which the normal is stated to be five and one-half minutes at 25° C. His results showed coagulation times varying from thirteen minutes to two and one-half hours, and the author concludes that among hemophiliacs the coagulation time is an individual characteristic that shows wide variations. Reports from other investigators indicate in general a longer time than is given in Schloessmann's figures. In the cases that I have examined the coagulation time has never been less than an hour and reports in the literature vary usually from about an hour to as much as twelve hours or more.

For any one individual the coagulation time is subject to irregular changes for reasons that are not apparent. Schloessmann[3] believes that these variations are practically of little importance and for the most part fall within the limits of error of the method. But this has not been my experience and his view is not I think supported by reports from other workers. With three of my cases I have been in quite close contact for a period of about twenty-five years, and during that time I have had many occasions to determine the coagulation time. All three have shown variations that lie well beyond possible limits of error of the method. In one of these cases who has served as a laboratory assistant a record was kept of 150 determinations extending over several years. The times of coagulation varied from about one hour to four hours, with an average of two hours and twenty minutes. Feissly's[12] tables show more extensive variations and in the curves for seven patients published by Stetson, Forkner, Chew and Rich,[13] covering a period of a few months, variations as much as from one to twelve hours are noted. In my own cases I have not been able to satisfy myself that spontaneous attacks of joint bleedings, which have occurred at irregular intervals, were associated with the times of slower coagulation. Whatever the condition may be that is responsible for the retarded coagulation of hemophilic blood it is evidently subject to frequent and considerable variations. It is conceivable therefore that at times the process might approach or reach a normal period, but as has

been pointed out there is no good evidence that this ever occurs. The time of coagulation is always in excess of the normal.

All recent experimental investigations have been concentrated upon an effort to determine the factor or factors responsible for the retarded coagulation. There seems to be no question that the fibrinogen in hemophilic blood is present in normal amount and exhibits normal properties; on addition of active thrombin the blood clots promptly. There is general agreement that the delay in clotting is due to some abnormality in the factors or processes concerned in the formation of thrombin. Theoretically the delay might be due to the existence of an inhibitory substance or to some change in quantity or properties of the three known thrombin factors, prothrombin, calcium and thromboplastic substance. All of these hypotheses have been made by one worker or another and each has supported his views by experimental evidence. Time does not permit me to refer specifically to all the theories that have been proposed. The one that has, perhaps, met with most approval and support by workers, in this country, at least, is that advanced by Addis.[14] His experiments led him to suggest that the prothrombin is at fault. While present in normal amount it is qualitatively different in that it is less reactive and hence is converted to thrombin more slowly. The experiments published by Addis in support of this hypothesis while seemingly conclusive are, I believe, open to criticism. Prothrombin precipitated from hemophilic plasma by CO_2 was found to be less reactive than similar preparations from normal plasma. But the CO_2 method precipitates from plasma fibrinogen and thromboplastic material as well as prothrombin. In Addis's experiments the fibrinogen was removed but the thromboplastic substance was left. The effect of this latter substance on the clotting time was ignored on the assumption that it exists in equal amounts in normal and hemophilic plasma. This assumption is not justified as has been abundantly shown by recent work which will be referred to later. Addis was led into error on this point because his previous experiments had convinced him that in normal and hemophilic serum the amounts of thromboplastic substance are the same, but proving this point for serum does not establish it for plasma. In the serum of hemophilic blood the platelets have all disintegrated and yielded their quota of thromboplastic material. It is now known that the plasma of hemophilic blood contains less thromboplastic substance than that of normal blood. If equal amounts of the two plasmas are diluted and precipitated by CO_2 more thromboplastic substance

comes down with the prothrombin in the normal blood and this fact is sufficient explanation why the prothrombin is activated more promptly by calcium than in the case of hemophilic blood. The same criticism applies to Eagle's[15] confirmation of Addis's results, since his prothrombin was prepared essentially by the same method.

Making use of the acetone method, Howell and Cekada[16] were not able to find any significant difference in quantity or reactivity between prothrombin from normal blood and that from hemophilic blood, and Frank and Hartmann[17] report that prothrombin (proserozyme) from hemophilic blood clots a fibrinogen solution (phosphated plasma) as promptly as prothrombin from normal blood. Nevertheless normal blood contains something, other than prothrombin, which is lacking or deficient in hemophilic blood. Frank and Hartmann emphasize a fact which had been noted previously by Sahli[6] and others, namely, that hemophilic blood can be made to clot promptly if normal human plasma is added to it in small amounts, even when the normal plasma has been deprived of its prothrombin by the action of calcium phosphate. They gave to this result the interpretation that normal plasma contains an activator of some kind which facilitates the formation of thrombin and which is absent or deficient in amount in hemophilic blood.

More recently this reaction has been investigated in this country by Patek and Stetson,[18] Patek and Taylor and Pohle and Taylor[19] and in Holland by Bendien and Van Creveld.[20] Starting with the fundamental fact that normal citrated blood added to hemophilic blood, even in quite small quantities, reduces the coagulation time of the latter to the normal range, for example from 170 minutes to 15 minutes, these workers have demonstrated that the active substance concerned can be isolated by precipitating the plasma, after dilution, by acid added to a pH of 5.3 to 5.8. Extracts of this precipitate, before or after drying, in physiological salt solution when added to hemophilic blood cause a marked shortening of its coagulation time. If a hemophilic plasma is treated in the same way a similar precipitate is obtained but its extracts have a distinctly smaller effect in accelerating the clotting of hemophilic blood, showing that the hemophilic plasma contains less of the active substance. Solutions of the active substance thus prepared give protein reactions (they may in fact contain some prothrombin or fibrinogen) and Patek[18] assumes that the active material is a protein body of a globulin nature, which he designates as "globulin substance" and which is closely associated with prothrombin.

Bendien and Van Creveld[20] obtain essentially similar results; they give the name of coagulation globulin to the active material and believe that it is associated closely with fibrinogen, although they state that the globulin is to be considered only as a carrier of the active material, since when hemophilic blood is treated in the same way a globulin fraction of a similar amount is obtained which has but little activity in promoting the clotting of hemophilic blood. Experience has shown that this globulin substance or coagulation globulin when injected intravenously into a hemophiliac is effective in causing a reduction in clotting time similar to that obtained from transfusions of normal blood. According to Pohle and Taylor,[19] the same or a similar result may be obtained from intramuscular injections, but Bendien and Van Creveld[20] report that they could get no therapeutic effects from their solutions when given intramuscularly or when fed by mouth.

All this recent work furnishes, therefore, good evidence for the view that blood plasma contains a specific substance which accelerates the process of clotting and which is present in smaller amounts in hemophilic blood. It seems entirely probable that the deficiency of this material in the plasma of hemophilic blood is responsible for its delayed clotting and it becomes important to learn as much as possible about its nature and properties and its origin and fate in the body. I have been engaged for the past two years in work of this kind and would like to present briefly some of the results obtained. For the sake of convenience it is desirable to give the substance a provisional name until such time as its chemical nature is determined. The names already suggested, globulin-substance or coagulation-globulin seem to me to be inappropriate since they imply that it is protein in nature and, as I shall show later, this is not correct. The substance in fact is essentially the same material as that designated by Morawitz as thrombokinase. They have the same distribution and properties, such as loss of activity on standing, and probably the same mode of action, but this term carries the significance given to it by Morawitz of a heat labile protein with enzyme-like properties. Among other names suggested hitherto to designate the material in the blood or tissues which accelerates the formation of thrombin, that of thromboplastin is perhaps the most appropriate, since it is a convenient and non-committal abbreviation for the phrase thromboplastic substance. Hereafter in this paper I shall speak of this as plasma thromboplastin and designate the similar or identical substance occurring in the tissues as tissue thromboplastin.

Plasma thromboplastin, as we have seen, may be obtained in crude form from oxalated or citrated plasmas, after dilution, by saturating with CO_2 or by adding acid to a pH of about 5.5. The precipitate obtained contains prothrombin and fibrinogen as well as thromboplastin, but the activity of its extracts in clotting hemophilic blood is not due to either the prothrombin or the fibrinogen. Extracts may be made which are active but which contain neither prothrombin nor fibrinogen—glycerol extracts for example. Moreover the substance may be prepared equally as well from plasma in which the prothrombin has been removed by treatment with magnesium hydroxide or calcium phosphate, or from plasmas in which the fibrinogen has been removed by heat coagulation at 56° C. It is easy to show that not all of the plasma thromboplastin is removed by precipitation with acid. The plasma that is left, when neutralized, still shows the property of hastening the coagulation of hemophilic blood. If the globulins of the plasma are fractionated by precipitation with ammonium sulphate at various concentrations, 25 per cent, 33 per cent, 50 per cent and finally the serum albumin by addition of acid, all the precipitates on examination will show the presence of some plasma thromboplastin. It seems to be associated with, or, at least, be precipitated with all the proteins of the plasma although most of it in normal plasma comes down with the fibrinogen and the euglobulin fraction. If fibrinogen is prepared from normal plasma by precipitation with ammonium sulphate added to a concentration of 25 per cent, instead of by acid, the precipitation being repeated several times, the final solution of purified fibrinogen in physiological saline has a marked accelerating effect upon the clotting of hemophilic blood. If the fibrinogen in such a solution is removed by heating to 56°C., the solution remaining after filtration shows the same coagulating action upon hemophilic blood. If the fibrinogen is associated in any way with the thromboplastin, the union is broken when the fibrinogen is denatured and precipitated by heat.

My experiments corroborate those of previous observers in indicating that plasma thromboplastin is present in smaller amounts in hemophilic than in normal blood. In the cases that I have used in my experiments the difference was not so great as has been noted by other observers, but it was very distinct and invariable when the concentrations used were not so strong as to obscure the relations. Hemophilic blood is not entirely lacking in thromboplastin, but it contains less of it. The difference in this respect between it and normal blood might well be used as an addi-

tional diagnostic test. The method used was to oxalate equal amounts of normal and hemophilic blood and remove the corpuscles by centrifuging. The clear plasma was diluted ten times with distilled water and was then precipitated by CO_2 or by the addition of dilute acetic acid. The precipitates, obtained by centrifuging, were washed with distilled water and then extracted with physiological saline made neutral or slightly alkaline with sodium bicarbonate. With solutions prepared in this way such comparative results as the following were obtained:

1. Hemophilic blood 0.5 cc. + thromboplastin extract of normal plasma 0.3 cc., clot in three minutes.
   Hemophilic blood 0.5 cc. + thromboplastin extract of hemophilic plasma 0.3 cc., clot in eight and one-half minutes.
   Control. Hemophilic blood 0.5 cc. + physiological saline 0.3 cc., clot in ninety-five minutes.
2. Hemophilic blood 0.5 cc. + thromboplastin extract of normal plasma 0.3 cc., clot in four minutes.
   Hemophilic blood 0.5 cc. + thromboplastin extract of hemophilic plasma 0.3 cc., clot in twelve and one-half minutes.
   Control. Hemophilic blood 0.5 cc. + physiological saline 0.3 cc., clot in eighty-five minutes.
3. Hemophilic blood 0.5 cc. + thromboplastin extract of normal plasma 0.3 cc., clot in 19.5 minutes.
   Hemophilic blood 0.5 cc. + thromboplastin extract of hemophilic plasma 0.3 cc., clot in 47.5 minutes.
   Control. Hemophilic blood 0.5 cc. + physiological saline 0.3 cc., clot in ninety minutes.

In the last experiment the extracts were less concentrated and were prepared from magnesium sulphate plasma instead of oxalated plasma.

The difference between the two bloods in respect to their content in thromboplastin was brought out also when a fibrinogen solution was prepared from equal quantities of each by precipitation with ammonium sulphate. In each case three precipitations were used and the final solution of fibrinogen was tested for its thromboplastin content upon hemophilic blood. The fibrinogen solution from normal plasma caused clotting of hemophilic blood in a few minutes while that from hemophilic plasma had little or no action. In one such experiment, for example, the control with saline clotted in fifty-seven minutes, the fibrinogen solution from normal plasma caused clotting in fourteen minutes and that

from the hemophilic plasma in forty-eight minutes.

Saline solutions of the plasma thromboplastin obtained by the method described always contain protein, but the protein is not concerned in, or at least, is not essential to the clotting reaction, since solutions of thromboplastin showing its characteristic reaction on hemophilic blood may be obtained free from protein. The simplest method for this purpose is extraction with glycerol. The precipitate obtained from diluted plasma by the addition of acid, after washing with distilled water to remove adherent plasma, is stirred in glycerol, allowed to extract for twenty-four hours in the ice box and then filtered. The extract is free from protein as far as can be determined by the ninhydrin and biuret reactions. In testing its effect on hemophilic blood it was usually diluted with two volumes of a 1.5 per cent solution of sodium chloride and its effect compared with that of a control solution consisting of one part glycerol and two parts 1.5 per cent solution of sodium chloride. Glycerol itself in this dilution accelerates slightly the time of clotting, compared with an equal volume of physiological saline, but far less than the glycerol extract. A typical result is, as follows: Hemophilic blood 0.5 cc. + glycerol extract of thromboplastin 0.3 cc., clot in 14 minutes; hemophilic blood 0.5 cc + glycerol-saline solution 0.3 cc., clot in 51 minutes. The solubility of the coagulin in glycerol must be small since it does not seem possible to get concentrations that have as strong an action as the saline extracts. I have obtained plasma thromboplastin free from protein also by precipitating the protein in the extracts with petroleum ether. The method is uncertain and is successful only when the amount of contaminating protein is small. I have referred to the fact that when fibrinogen is prepared from plasma by the ammonium sulphate method it always contains some thromboplastin. If the fibrinogen is removed by heating to 56° the thromboplastin is left in solution together with a small amount of an unknown protein which undergoes heat coagulation at 75° to 80°C. If a solution of this kind, from which the fibrinogen has been removed, is shaken with two volumes of petroleum ether a gelatinous precipitate forms. If this is removed and dried and extracted with physiological saline, a solution is obtained which is protein free but which accelerates distinctly the clotting of hemophilic blood. The petroleum ether used contains none of the active substance.

Blood plasma contains only small amounts of thromboplastin and it did not seem feasible to obtain it protein-free from this source in suffi-

cient amounts to make any progress in determining its chemical nature. I have therefore turned to a study of the tissue thromboplastin. It has long been known that the tissues contain a thromboplastic substance which accelerates the clotting of blood and a number of workers have called attention to the fact that this substance is found in especially high concentration in the lungs. Specimens of hemophilic blood which require one or more hours before coagulation begins will set to a firm clot in less than a minute when mixed with a small amount of lung extract. Numerous attempts have been made to utilize extracts of the lungs or other tissues in the control of hemophilia or as a hemostatic agent in general. Preparations of this kind have come upon the market under trade names such as the coagulen of Kocher-Fonio, the clauden of Fischl, fibrinogen-Merrell of Mills, thromboplastin. The therapeutic value of these agents, used intravenously or intramuscularly, has been reviewed critically by Gold.[21] The results from their use in the hands of different observers seem to be contradictory and are not unattended by danger from protein reactions or intravascular clotting. Nevertheless, there is no question that in the tissues and especially in the lung tissue there is a substance which has a remarkable effect in accelerating the clotting of hemophilic blood *in vitro*. It would seem that we should be able to obtain this substance in pure form and to utilize it in controlling hemophilic bleeding.

The active substance as usually obtained is associated or combined with protein, but by a modification of the method of extraction I have succeeded in obtaining it in a protein-free condition and in a form suitable for chemical examination. The details of the method will be given in a later publication. The essential point is that the acid precipitate of the aqueous extracts of the lungs, after purification by repeated precipitations, is extracted with distilled water in large volume and this dilute solution or suspension is precipitated by the addition of acid to a pH of about 4. The precipitate is obtained by centrifuging and is further purified by extraction with chloroform. The yield is small, about 10 to 15 mgm. per gram of dried lung tissue, but the method no doubt can be improved in this respect. The material obtained is soluble in slightly alkaline aqueous or saline solutions and shows great activity in accelerating the clotting of hemophilic blood, an activity of the same order as that of extracts of lung tissue. It shows the following properties. It is apparently insoluble in chloroform, ether and benzol and its activity is de-

stroyed by the action of alcohol or acetone. It does not contain a carbohydrate group. It gives positive reactions for phosphorus and nitrogen. Fused with acid potassium sulphate it gives reactions for glycerol and one may assume that it contains a glycerophosphate group. It is interesting to note in this connection that Freund[22] in 1910, in a report made to the International Physiological Congress, stated that by hydrolysis of lecithin he obtained a substance that he believed to be a calcium diolein glycerophosphate which showed marked thromboplastic activity. It is possible of course that the material, as I have isolated it, may contain substances other than the thromboplastin. This must be determined by further work.

The thromboplastic material of the tissues has been investigated by many observers. As is well known two kinds of extracts have been made; aqueous or saline extracts which yield a very active thermolabile compound, containing protein, the thrombokinase of Morawitz, and alcohol-ether extracts which are less effective and in which the active constituent is believed to be the phospholipid, cephalin. Extracts of the latter kind yield the zymoplastic substance of Schmidt and the cytozyme of Bordet. It has been suggested by Howell, Mills and others that cephalin constitutes the active component in water extracts also, on the theory that in the tissues it is in combination with protein and in this form exhibits thermolability as well as greater potency. The work here reported, however, does not support this view. It indicates on the contrary that the active substance in aqueous extracts, of the lung tissue, at least, is not a known phospholipid, although it may possibly be a derivative product.

In the lungs the thromboplastin is associated apparently with a nucleoprotein and in blood plasma mainly with the globulins. While this association may modify its reactions it does not seem to enhance its thromboplastic activity since the protein-free thromboplastin is still remarkably potent.

As far as blood is concerned we may conclude that one real difference between normal and hemophilic blood is that the latter contains less thromboplastin in its plasma. There may be other differences but they have not been discovered. It would seem permissible to assume that this deficiency is sufficient to explain the prolonged clotting time of hemophilic blood, since clotting takes place normally when the deficiency is removed by the addition of thromboplastin extracts, *in vitro*, or *in vivo*, by the transfusion of the plasma of normal blood or the intravascular in-

jection of globulin substance. Important questions in regard to the plasma thromboplastin are what is its source and how does it affect the coagulation process. It is not possible at present to give wholly satisfactory answers to these questions, but some facts bearing upon them may be discussed to indicate the difficulties involved and the solutions proposed.

With regard to the source of the thromboplastin present in the circulating blood one view is that it is derived from the blood platelets. It has long been known that the platelets contain thromboplastic substance. I have had occasion to corroborate this belief in my own experiments. Platelets were obtained from normal and from hemophilic blood by the usual method and were thoroughly washed in a large volume of buffer-saline solution. When treated with a slightly alkaline saline solution they gave extracts which greatly accelerated the clotting of hemophilic blood *in vitro*. There was no difference in effect between the extracts of the normal and the hemophilic platelets. We have good reasons for believing that in the circulating blood the platelets are constantly going into solution. The platelets are not cells but pieces of the cytoplasm of the megakaryocytes that are given off to the blood. Donahue and I[23] have shown, by counts made upon arterial and venous blood, that in passing through the systemic capillaries there is a considerable loss in number which is made good by the addition of new platelets to the blood as it traverses the lungs. There is an established equilibrium between the production and destruction of the platelets and we may assume that the thromboplastic material contained in them is being constantly supplied to the blood. It seems probable that this process accounts for the presence of thromboplastin in the blood plasma.

This assumption or hypothesis brings up the interesting but difficult question as to whether there is any difference in structure or properties between hemophilic and normal platelets. The point has been much discussed in the literature on coagulation. To illustrate opposing points of view I may refer briefly to two rather recent contributions. Govaerts and Gratia[24] published experiments which they believe prove that when hemophilic platelets are transferred from their own plasma, in which they exhibit unusual stability, to normal plasma they react like normal platelets, that is to say, they disintegrate rapidly. They conclude, therefore, that there is no difference between the two kinds of platelets. The apparently greater stability of the hemophilic platelets is due to the absence or deficiency of some constituent of the plasma, and it follows that the

inherited defect in hemophilia lies in this abnormality in composition of the plasma. This conclusion may be correct but in one respect their experiment seems to me to be unsatisfactory. For a normal plasma they used a decalcified blood in which coagulation was induced by recalcification. What changes are caused by decalcification and recalcification are not fully known but plasma so treated can scarcely be regarded as an entirely normal environment for platelets. For the point at issue it would be a crucial experiment if uninjured hemophilic platelets could be transferred to a platelet-free normal human plasma without employing any decalcifying or anticoagulating agent. This has not been done and it is difficult to see how it could be accomplished. The reverse experiment of transferring normal platelets to a platelet-free hemophilic plasma is more feasible and has been attempted by Fonio.[25] In his experiments hemophilic blood was received into paraffined centrifugal tubes packed in ice which were then centrifuged at high speed and at a low temperature. In this way he obtained a platelet-free hemophilic plasma which at room temperature clotted very slowly in from four to eight hours. If to this plasma he added suspensions of washed platelets from normal and from hemophilic blood the clotting was greatly accelerated but much more in the case of the normal than in that of the hemophilic platelets although the final result was the same for both. Here apparently under the same conditions the platelets behaved differently, those from hemophilic blood showing greater stability. There was nothing in the hemophilic plasma to retard the breaking down of the normal platelets. From this point of view the inherited defect in hemophilia would lie in the structure of the platelets or of the megakaryocytes from which they are derived. In a former publication I have advocated a similar view, but it must be admitted that such a theory is at variance with the conclusion to be drawn from the newer work that I have been describing. The point is this. When blood is transfused into a hemophilic patient, or globulin substance is injected, the thromboplastin content of his plasma is increased but not enough to cause intravascular clotting. We may assume that in this respect the hemophilic plasma is brought to an approximately normal condition. If now this blood is withdrawn from the circulation it clots normally. The hemophilic platelets presumably agglutinate and break down in what we may consider the normal time, that is to say, when the plasma is right they exhibit no abnormal stability.

The part taken by the thromboplastic substance of the blood in

clotting is a difficult and controversial subject which I shall not attempt to discuss at length. It may be said that thromboplastin when present in sufficient concentration causes coagulation of the plasma without participation by the platelets. Platelet-free hemophilic plasma obtained by centrifuging the blood at high speed in paraffined tubes clots solidly in one to two minutes on the addition of a solution of purified thromboplastin prepared from lung tissue, and it has long been known that rapid intravenous injections of tissue extracts cause almost immediate intravascular clotting. It would seem, therefore, that in the circulating blood the content of thromboplastin must lie below the concentration required to induce prompt clotting. When blood is shed or withdrawn, the content of thromboplastin is increased with some suddenness, by the mass disintegration of the platelets, to a concentration sufficient to cause clotting within a few minutes; the actual time varying with conditions such as temperature or contact with a foreign surface. A point of interest is whether or not the thromboplastin has any direct influence upon the agglutination and breaking down of the platelets. I have no conclusive data upon this point but I am inclined to believe that thromboplastin does not affect the platelets directly although it may do so indirectly by setting up certain chemical changes in the plasma. Platelets when removed from their normal environment by centrifuging and washing exhibit a marked rigidity in structure. In saline or even aqueous suspensions they retain their shape, do not agglutinate and show no tendency to break down upon contact with glass or other foreign surface. Addition of solutions of thromboplastin to such suspensions does not seem to affect the platelets in any way. Yet in shed blood, platelets do agglutinate and disintegrate quickly, and when the concentration of thromboplastin is subnormal, as in the case of hemophilic blood, the process is markedly delayed, indicating that the thromboplastin is concerned in some way in the changes undergone by the platelets. These contrary indications are difficult to reconcile, but it is clear, I think, that the primary function of the thromboplastin is to participate in the activation of prothrombin to thrombin. In shed blood, both physical and chemical factors may accelerate this reaction and thereby alter conditions so as to affect the platelets. For example, platelet-free or platelet-poor hemophilic blood in mass in a glass container clots very slowly and much more slowly if the walls of the container are paraffined. But if a sample of this plasma is placed in a counting chamber and observed under the microscope it will be found

that clotting begins in a few, five to ten, minutes. Needles of fibrin are deposited in the plasma quite independent of any platelets that may be present and they increase rapidly in number until a meshwork of fibrin is formed. The greater physical contact with the glass surface in this case seems in itself to accelerate the chemical changes leading to the formation of thrombin, and it is possible that some product of this reaction, in which thromboplastin is concerned, may affect the integrity of the platelets, in addition to the well known influence of contact with a foreign surface. However these are speculations which serve mainly to emphasize some of the problems that need further study.

In conclusion something may be said in regard to the therapeutic treatment of hemophilia. What we can hope for is some method of controlling promptly internal and external hemorrhages, for there is little reason to believe that an hereditary defect of this kind can be permanently cured by treatment. External capillary hemorrhages from small wounds or abrasions of the skin are not usually serious, since the admixture of the tissue juices is sufficient to ensure clotting. In wounds of larger vessels the ordinary surgical methods of closing the wound may be supplemented by transfusion and by the application of freshly prepared tissue extracts. I have lately found that glycerine extracts of dried lung give an unusually potent preparation that might be used for such a purpose. It has the advantage that it retains its activity for a long period if kept in the ice box. The more serious problem is the control of the internal hemorrhages. The customary method used for this purpose is the transfusion of normal blood. In the majority of cases reported this method has proved to be efficacious. The curves given by Patek and Stetson[18] show that after such transfusions the clotting time of the hemophilic blood falls quickly toward or to the normal and may remain at this level for a number of hours or for a day or two. It then returns somewhat rapidly to its former condition. Since a similar effect is produced when blood plasma alone is used it may be assumed that the result is due not to the corpuscles but to some constituent in solution in the plasma. Normal plasma, as we have seen, differs from hemophilic plasma, so far as our positive knowledge goes, only in having more thromboplastin, and we may conclude therefore that the good effect of the transfusion is due to the fact that it increases the concentration of thromboplastin in the hemophilic blood. This conclusion is supported by the direct experiments of Patek and Stetson[18] and of Pohle and Taylor[19] which

show that intravenous injection of their "globulin-substance" causes a shortening of the coagulation time comparable to that produced by blood transfusions. It may be that there are other factors or conditions involved which influence or modify the effect of the thromboplastin. Some facts, at least, are known which indicate that the time of coagulation does not always vary directly with the concentration of thromboplastin in the blood. It is stated, for instance, by Feissly[12] that transfusions of as little as 10 to 20 cc. of normal blood suffice to bring the coagulation time of hemophilic blood within normal limits. Patek and Stetson[18] also report that transfusions of 30 to 40 cc. of citrated blood may have the same effect as much larger amounts, and Pohle and Taylor[19] found that in a patient subjected to repeated injections of globulin substance, at six hour intervals, a refractory state may develop during which the coagulation time seems not to be affected by an increase in the substance in the plasma. However this may be, we have the practical result that the coagulation time of hemophilic blood may be restored to normal by injection of solutions of thromboplastin. Experience must determine whether this substance in purified form may be used as a safe substance for blood transfusion.

Many other methods of controlling hemophilic bleeding have been suggested but the claims made for their efficacy have not in most cases been confirmed by subsequent experience. It is not possible to refer to all of these methods but a word or two may be said about some that have been recently proposed. Birch[26] proceeding upon the theory that the hemophiliac is lacking in the female sex hormone has treated cases successfully with ovarian extracts and estrone injections. Her results have been confirmed by some observers and denied by others. I have not myself been able to detect any positive effect of injections of theelin upon the coagulation time of hemophilic blood. The careful study of seven cases by Stetson and his co-workers[13] seems to demonstrate conclusively that the feeding or injection of ovarian extracts and estrogenic preparations has no distinctly beneficial effect upon hemophilic bleeding or the coagulation time of the blood.

Timperley, Naisch and Clark[27] report upon a new material which they obtain by incubating a mixture of egg white and potassium bromide. The chemical nature of the active substance derived from this mixture was not known, but its use intravenously gave most interesting results. There was no reaction, the coagulation time of the blood was reduced

to normal and if the injections were made during an attack they brought relief to the patient. Unfortunately these results have not met independent confirmation from other workers, and the authors themselves have not so far been able to devise a reliable method for obtaining the effective substance.

Lawson and Graybeal[28] report good results from simply bleeding the patient. Blood in amounts from 170 cc. to 500 cc. was withdrawn by syringe at the time of attacks with the result that the patient showed marked improvement and disappearance of symptoms. In one case bleeding was done periodically over a stretch of seven years, 500 cc. being withdrawn every six to eight weeks. They report that during this period there was a cessation of serious attacks which before had been frequent. The authors do not state the effect of these bleedings upon the coagulation time. It was a former belief, based upon Sahli's[6] work, that severe hemorrhage in the hemophiliac causes a reaction of some kind that affects the coagulation of the blood, bringing it back to normal, thus affording a mechanism for automatically controlling the hemorrhage, but later observers (Schloessmann[3]) have not been able to confirm this result.

Many attempts have been made also to control the coagulation time in hemophilia through the diet or by extracts administered through the mouth. These attempts so far as I know have been uniformly unsuccessful. Some years ago I tested a number of these methods upon one of my cases. The series consisted of Nateina tablets, Llopis, a mixture of vitamins A, B, C, D, with calcium phosphate and milk sugar; ceanothyn, the mixed alkaloids of Ceanothus Americanus; cephalin, a crude extract from brain in aqueous suspension, Howell; a liver and grapefruit diet, Pickering; spleen and bone-marrow extracts, Leake; ovarian extracts and theelin, Birch; placental extracts, Eley; extracts of fresh lung; extracts of dried lung; brewers' yeast; cod liver oil; hemostyl, serum hemopoietique, Roussel; rivanol; hexylresorcinol; carbon tetrachloride; sodium bicarbonate, alkalosis; ammonium chloride, acidosis; fasting for a period of forty-eight hours. None of these substances had a definite effect. At times there might appear to be a shortening of coagulation time; never a return to normal although a considerable diminution, but on repetition uniform results could not be obtained, the effect was inconstant. I was forced to conclude that such variations as occurred were due to spontaneous changes in the coagulation of the blood or to small differences in the technique

of sampling. During the present year I have tried the effect of administering by mouth solutions of purified thromboplastin, obtained from lung, which *in vitro* caused hemophilic blood to clot within one or two minutes, but the results were entirely negative. It would seem to be impossible to affect the coagulation time of the blood in a definite way by absorption from the alimentary canal. For the control of hemophilic hemorrhages we must rely at present chiefly upon transfusions, but there is a prospect that further study of the chemistry and reactions of thromboplastin may provide a more convenient treatment in which the dosage may be regulated to meet the severity of the symptoms.

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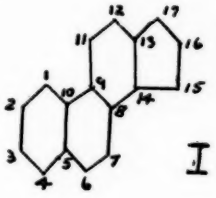
SOME ASPECTS OF THE INTERMEDIARY METABOLISM OF THE STEROID HORMONES*

Harvey Lecture, October 20, 1938

GUY FREDERIC MARRIAN

I MUST begin by reminding you that the term "steroid" has now come into general use, following the suggestion of Callow and Young,[1] as a group name for all substances containing the cyclopentenophenanthrene carbon skeleton (I).



I

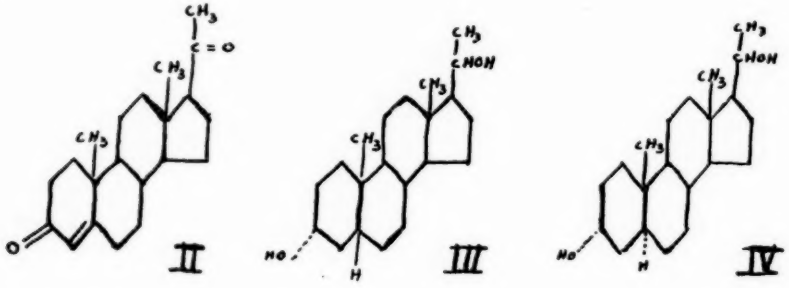
Among the large number of interesting naturally occurring compounds which possess this carbon skeleton are four groups of hormones: the estrogens elaborated by the ovary and also possibly by the placenta, the androgens of the testis, the progestational hormone of the corpus luteum, and the "life-maintaining" hormone or hormones of the adrenal cortex. These hormones may therefore be conveniently called "steroid hormones."

During the past ten years interest in these hormones has been largely centered round the problems of their exact chemical constitutions and of their respective physiological functions in the animal body. We are now, I believe, at the beginning of a new phase in research upon these steroid hormones, a more purely biochemical phase, arising out of and made possible by the achievements of the organic chemists and physiologists during the past decade, the ultimate object of which is the elucidation of the chemical mechanisms involved when these hormones produce their physiological effects in the organs and tissues upon which they act. Already in a number of different laboratories, studies have been made upon the

* From the Department of Biochemistry, The University of Toronto.

nature of the chemical reactions involved when certain of these hormones are inactivated in or eliminated from the body; and speculating upon their possible modes of origin and interconversion is rapidly becoming the fashionable biochemical pastime.

So rapidly has this field expanded during the past few years that the task of reviewing it critically and comprehensively is an impossible one in the short time at my disposal. I must therefore regretfully limit myself mainly to an account of some of the problems in which I and my colleagues are at present interested, mentioning the results and theories of others only when they are strictly relevant.



It will be convenient to begin by mentioning some of the well established facts about metabolic changes undergone by the hormone of the corpus luteum, progesterone (II).

It is a point of some historical interest that a metabolic reduction product of progesterone had been isolated and chemically characterized five years before the hormone itself was isolated in a crystalline form. This compound, pregnanediol (III), was first isolated by myself[2] in 1929 in a slightly impure state from human pregnancy urine. A few months afterwards it was independently isolated by Laqueur and his co-workers[3] and by Butenandt[4] from the same source. The latter was soon able to establish the structural relationship of this compound to the bile acids with complete certainty.[5]

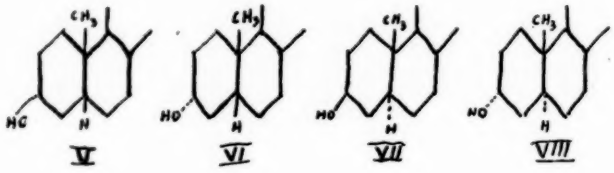
The true significance of the presence of this steroid in pregnancy urine could not at that time be appreciated. However, as soon as the constitution of progesterone had been established in 1934, it was immediately seen that pregnanediol might be a metabolic product of the hormone, formed by the reduction of its two ketonic groups and of its double linkage. The discovery by Hartmann and Locher[6] in human pregnancy

urine, of a stereoisomer of pregnanediol, allopregnanediol (IV), which differed from the former in its steric configuration at C_5, provided support for this view, since the presence of both C_5 stereoisomers in urine implied that both must have been formed in the body by the addition of hydrogen to a double-linked (and therefore non-asymmetric) C_5 of a common precursor. Conclusive proof that pregnanediol is indeed a metabolic product of progesterone has been recently put forward by Venning and Browne.[7] They were able to show in the first place that pregnanediol is present in human pregnancy urine in combination with glucuronic acid. This observation, interesting enough in itself, assumed great practical importance when it was found[8] that the solubilities of the sodium salt of this pregnanediol glucuronic acid conjugate were such that it could be quantitatively determined in pregnancy urine with a fair degree of accuracy by a relatively simple procedure. Using this method of determination, they were successful in demonstrating an increase in pregnanediol excretion in a non-pregnant woman following the administration of progesterone.[9] We are therefore fully justified in recognizing pregnanediol as an end product of the metabolism of progesterone, and it follows that the same may be said of allopregnanediol. As far as I am aware, there are no reliable figures in the literature to indicate the relative amounts of these two end-products of progesterone metabolism which are excreted in the urine. My own experience with pregnancy urine extracts, however, leaves me in no doubt whatsoever that the reduction of progesterone in the body leads to the formation of much more pregnanediol than allopregnanediol. As we shall see later, it is possible that this fact may not be devoid of biological significance.

As might be expected, pregnanediol and allopregnanediol are not the only simple reduction products formed from progesterone in the body. Many partly reduced intermediates are theoretically possible, and since the addition of hydrogen to the three reducible groups of progesterone involves the formation of three new asymmetric carbon atoms, a very large number of products might result from the simple reduction of the hormone. Several of these have indeed been isolated from human pregnancy urine by Marker and his collaborators.[10,11,12]

Before proceeding any further, I must digress for a moment in order to explain the particular system of nomenclature which I intend to use when I am referring to stereoisomers in this series of compounds. If the C_3 hydroxyl group in a particular compound has the same steric config-

uration as that of the hydroxyl group in cholesterol, which is assumed to be *cis* to the C_{10} angular methyl group, it is designated by the prefix β-. Thus the partial formulae (V) and (VII) are of 3β-hydroxy compounds. Such compounds are precipitated by digitonin. When the C_3 hydroxyl group has the epimeric configuration as in the partial formulae (VI) and (VIII), the prefix α- is used. Such compounds are not as a general rule precipitated by digitonin. The prefix *allo-* is, as I have already implied, used to designate the steric configuration at C_5. Thus in accordance with this system of nomenclature, the compounds which I have been somewhat loosely referring to as pregnanediol and allopregnanediol, and which are of types (VI) and (VIII) respectively, should be called pregnane-3(α), 20-diol and allopregnane-3(α), 20-diol.



Some years ago, before the relationship between pregnanediol and progesterone had been discovered, Dr. Haslewood and myself were attempting to show the presence of the former substance in the urine of pregnant mares. We failed to do so, but we succeeded in isolating another interesting substance which we suspected might be closely related to prenanediol.[13] This compound gave analyses in close agreement with those required for a triol of the formula $C_{21}H_{33}(OH)_3$, but since we were unable to make any molecular weight determinations we were unable to go further than stating that it had the empirical formula $(C_7H_{11}OH)n$.

Within the last year Marker and his associates[14] have also isolated this compound from the urine of pregnant mares, and more recently[15] have published results which they claim prove that this compound is pregnane-3(α), 4(β), 20-triol. They suggest that this compound is formed from progesterone by the reduction of both ketonic groups and the addition of the elements of water to the double linkage in the manner shown in Fig. A.

This view of the structure of the compound is one with which I cannot agree. Dr. Odell and I[16] have been working on the constitution of this compound for the last three years. Our evidence on its structure is not

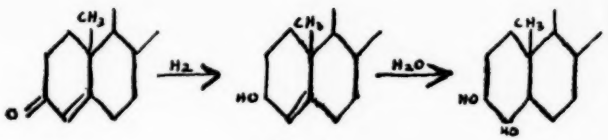


Fig. A

yet complete, but the data which we have so far obtained is absolutely irreconcilable with the conclusion of Marker *et al.* If the compound is indeed a steroid with three hydroxyl groups, two of which are at positions 3 and 20—and of this we ourselves have no proof at present—then our evidence indicates that it is very probably a pregnane or allopregnane-3(α), 6,20-triol. The formation of such a compound from progesterone could be explained on the reasonable supposition that after the reduction of the C_3 ketonic group, and before the addition of the elements of water, the double linkage shifts from the 4.5 to the 5.6 positions as shown in Fig. B.

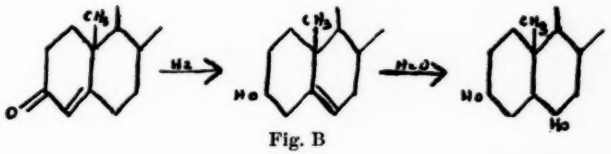


Fig. B

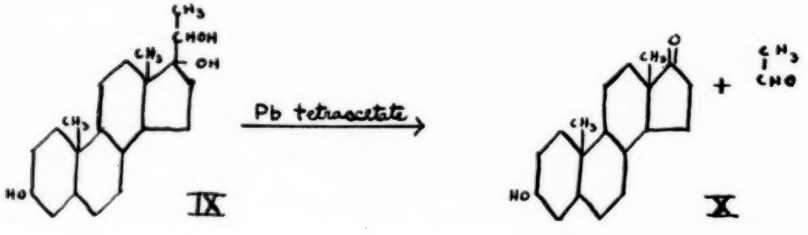
At the present time the biochemical significance of the presence of this compound in the urine of pregnant mares is not at all clear. At first we were inclined to believe that it represented the main end-product of progesterone metabolism in the mare, occupying a position in the metabolic scheme analogous to that occupied by pregnanediol in the human. However, since it has recently been shown by Marker *et al*[17] that pregnant mares' urine contains as much pregnanediol and allopregnanediol as does human pregnancy urine, this view must clearly be abandoned.

Although the position of this interesting triol in steroid metabolism is still obscure, there are no doubts in my own mind concerning the importance of its position in our own work on steroids, since it has been responsible for the initiation of a new line of research which promises to lead us to a real understanding of certain phases of steroid metabolism. Our isolation of the triol from mares' urine, led me some years ago to look for it in human pregnancy urine. I failed to find it, but I was able to isolate another new compound, which for brevity I will refer to as *x*. We are at present

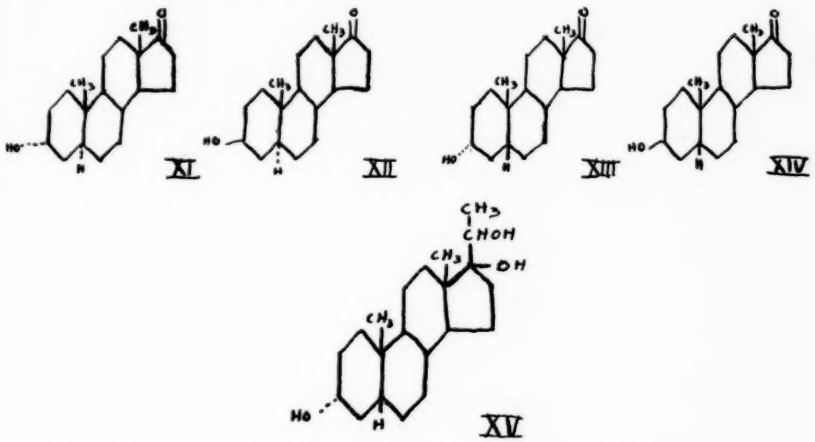
working on the constitution of *x*, but so far we have not progressed far enough to permit us even to hazard a guess concerning its structure, and therefore I am unable to make any speculations concerning its role, if any, in steroid metabolism. However, at one time I had the idea that *x* might be a metabolic product of certain of the adrenal steroids, and I therefore suggested to Dr. Butler that we might search for it in urine specimens obtained from individuals with hyperplastic adrenals. Mr. L. R. Broster of the Charing Cross Hospital, London, very kindly came to our assistance, and sent us several concentrates of urine obtained from women with marked symptoms of adrenal virilism and upon whom he subsequently operated. We were completely unsuccessful in isolating *x* from these urines, and I might add that we have now no grounds for believing that *x* is derived from the adrenal steroids as I had originally supposed. However, while we were looking for it, we found another new compound which almost certainly is an adrenal product, since we have never encountered it in any urines except those obtained from adrenal virilism cases.[18]

This new compound proved to be saturated triol of the formula $C_{21}H_{33}(OH)_3$—isomeric with but different from the triol in pregnant mares' urine. The elucidation of its constitution proved to be a surprisingly simple matter. We found that two of the three hydroxyl groups were easily esterifiable, whereas the third was not—a fact which suggested that the latter might be tertiary. We next indulged in some guessing. On the assumption that it was a steroid of the pregnane or allopregnane type with hydroxyl groups at positions C_3 and C_{20}, we guessed that the third hydroxyl group might be at C_{17}, by analogy with certain of the adrenal steroids, thus arriving at the hypothetical formula shown below (IX).

Such a compound should be capable of being oxidized by lead tetra-



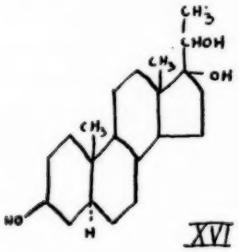
acetate to yield acetaldehyde and a hydroxyketone of the formula $C_{19}H_{30}O_2$ (X). Accordingly we attempted to oxidize our compound with this reagent. We found that it was oxidized, and in the products we identified acetaldehyde and a hydroxyketone which analyzed for $C_{19}H_{30}O_2$. It was apparent that if our original guess was correct, the latter must be androsterone (XI), isoandrosterone (XII), 3(α) hydroxyaetiocholane-17-one (XIII) or 3(β)-hydroxyaetiocholane-17-one (XIV). It proved to be identical with 3(α)-hydroxyaetiocholane-17-one. Consequently we had proved that the triol was pregnane-3(α),17,20-triol (XV).



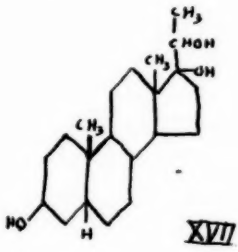
During the progress of this work we encountered in certain of the urine fractions small amounts of other crystalline substances. We therefore decided to attempt to work up much larger quantities of urine from adrenal virilism cases in order to obtain these other compounds in amounts sufficiently large for identification. Mr. Broster once again came to our assistance and sent us the concentrate from forty-five liters of urine collected from a female patient with adrenal hyperplasia and definite symptoms of virilism. The extract from this concentrate was systematically fractionated and yielded, as we had anticipated, several crystalline compounds, some of which we were able to identify with certainty.[19] The non-ketonic fraction yielded the same pregnane-3(α),17,20-triol, which we had previously isolated from the earlier urine specimens, while from the ketonic fraction we obtained two hydroxy ketones of the formula $C_{19}H_{30}O_2$. One of these was easily identified as isoandrosterone (XII) while the other proved to be 3(α) hydroxyaetiocholane-17-one (XIII).

Both of these substances were prepared by Ruzicka[20] from cholesterol in the course of his classical work on the constitution of pseudo-synthesis of androsterone. Neither of these compounds, however, had previously been isolated directly from any natural source. The identification of isoandrosterone is of some interest in connection with the etiology of adrenal virilism, since it possesses weak, but quite definite androgenic potency.

The presence in the same urine specimen of pregnane-3(α),17,20-triol and of 3(α)-hydroxyaetiocholane-17-one, the substance to which the former gives rise by oxidation with lead tetra-acetate, struck us as being very significant. It seemed probable to us that an oxidation similar to that which can be effected in the laboratory with lead tetra-acetate must have occurred in the body. Following this line of reasoning further, we decided that probably the isoandrosterone had arisen in a similar manner by an oxidative removal of the side chain of allopregnane-3(β),17,20-triol (XVI), which bears the same structural relationship to it as does pregnane-3(α),17,20-triol to 3(α)-hydroxyaetiocholane-17-one.



XVI



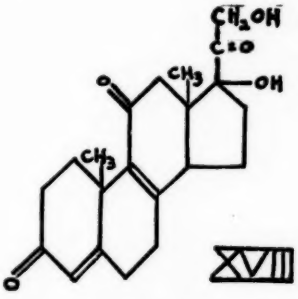
XVII

We accordingly conducted a search in our urine extracts for allopregnane-3(β),17,20-triol. We have so far failed to find it, but Reichstein[21] has recently reported the isolation from adreno-cortical extracts of two isomeric allopregnane-3(β),17,20-triols which must differ from one another in their steric configurations at C_{17} and/or C_{20}. Either of these could therefore give rise to isoandrosterone by a side-chain oxidation of the type that we suggest takes place in the body.

While we were conducting our abortive search for allopregnane-3(β),17,20-triol in urine, we isolated another triol. We had insufficient data for complete identification, but our evidence, such as it is, very strongly suggests that it is pregnane-3(β),17,20-triol (XVII). If our theory of side-chain oxidation is correct, then this triol should give rise to

3(β)-hydroxyaetiocholane-17-one (XIV) in the body. We have not so far been able to isolate this hydroxyketone from adrenal virilism urine. If we could do so, the evidence in favor of our theory of side-chain oxidation would be extremely convincing. As it is, the evidence is sufficiently strong in my opinion to justify its provisional acceptance as a basis for further work and speculation. It may be mentioned at this point that if our new triol is indeed pregnane-3(β),17,20-triol, its presence in urine will not be in line with the recent prediction of Marker[22] that saturated 3(β)-hydroxy steroids of the coprostane series will not be found in urine or tissues.

I must next discuss the problem of the mode of origin of these pregnane- and allopregnane-3,17,20-triols. Marker[22] has recently put forward the suggestion that the fundamental steroid from which all the adrenal steroids, the estrogenic and androgenic hormones are formed is the so far unknown $\Delta_{4,5,8,9}$-pregnadiene-17,21-diol-3,11,20-trione (XVIII), and which he suggests may be identical with the true adreno-cortical hormone.



XVIII

According to his theory, therefore, all these steroids are formed from a precursor which already possesses a C_{17} tertiary hydroxyl group. Dr. Butler and I hold a somewhat different view. We suggest that the steroids with C_{17} tertiary hydroxyl groups are secondary products which are formed by the addition of the elements of water to the double linkage of an enolized C_{20} ketonic steroid in the manner shown in Fig. C.

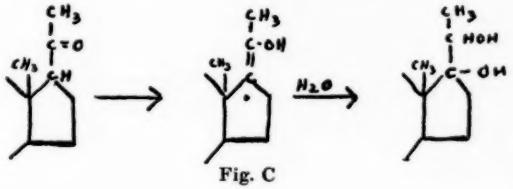
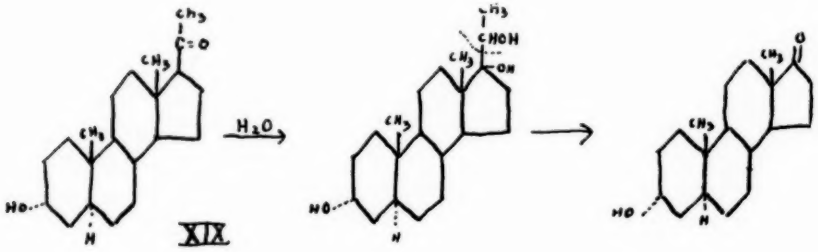


Fig. C

If this represents the true state of affairs and if our theory of side-chain oxidation is correct, then steroids with the allopregnane carbon skeleton, with C_3 hydroxyl or ketonic groups, and with C_{20} ketonic side chains attached to C_{17}, might be expected to have androgenic properties; since by the addition of water to the side-chain in the manner depicted above, and by subsequent oxidation between the so-formed C_{17} and C_{20} hydroxyl groups, androgenic compounds might be formed in the body. At the moment this is almost pure speculation supported by no experimental evidence of our own. Marker *et al*[10,23] have, however, made an observation which could be explained on the basis of our speculative theories, and which therefore provides them with some measure of experimental backing. From human pregnancy urine these workers isolated allopregnane-3(α)-01-20-one (XIX), one of the partial reduction products of progesterone, and they made the astonishing claim that it possesses powerful androgenic properties. We would suggest that the androgenic potency of this compound might be explained by supposing that it adds on the elements of water to the side-chain and is then oxidized with the formation of androsterone, according to the scheme shown below.



I have already pointed out that in reducing the double bond of progesterone the human body seems to have a preference for forming compounds of the pregnane rather than of the allopregnane type, as indicated by the fact that the pregnant woman excretes much more pregnanediol than allopregnanediol. This fact appeared to us to be somewhat significant in the light of our speculative theories, since the formation of an excess of reduction products of the allopregnane type might give rise to unwanted and harmful androgens by the series of reactions which we suggest may occur in the body.

In order to consider further the nature of the precursor or precursors

from which these isomeric triols arise, it will be convenient to make a list of the latter:

(a) pregnane-3(α),17,20-triol from urine,
(b) pregnane-3(β),17,20-triol from urine,
(c) two allopregnane-(β),17,20-triols from adrenals.

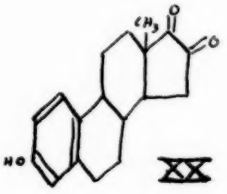
Now (a) differs from (b) and (c) in the configuration at the asymmetric C_3. It is therefore justifiable to assume that both C_3 stereoisomeric types have arisen from a common precursor with no asymmetry at C_3—that is to say probably from a compound with a C_3 ketonic group. Similarly (a) and (b) differ from (c) in the configuration at the asymmetric C_5. It therefore follows that both C_5 stereoisomeric types probably have arisen from a precursor with a double linkage at C_5. We are now in a position to attempt to identify this hypothetical precursor. Since it probably has ketonic groups at C_{20} and C_3 and probably has a doubly-linked C_5, it seems likely that it may be progesterone itself. Dr. Butler and I therefore believe that the various isomeric triols that are found in the urines of adrenal virilism patients and in the adrenal gland, are formed from progesterone in the adrenal cortex.

In support of our belief there is now a considerable weight of evidence that indicates that progesterone is manufactured in large amounts in the adrenal gland. In the first place Dr. Butler and I have observed that women with adrenal virilism symptoms excrete abnormally large amounts of pregnanediol, which, as has already been seen, is the chief end product of progesterone metabolism in women. Secondly, Callow and Parkes[24] have shown that extracts of the adrenals of certain species are highly potent in inducing progestational proliferation of the uteri of experimental animals. Finally, a few weeks ago, Beall and Reichstein[25] independently isolated pure progesterone itself from adrenal extracts. There can therefore be little doubt, I feel, that our contention that progesterone occupies a key position in steroid metabolism in the adrenal gland, is substantially correct.

Before I conclude, I wish to spend a short time describing some work along somewhat different lines which Mr. Darrach and I have been carrying out during the past two years and which promises eventually to throw much light on the intermediary metabolism of the estrogenic hormones. The original problem which Mr. Darrach and I set out to study was the purely academic one of the chemical mechanism of the well known Kober color reaction for estrogens. It will be recalled that this

color reaction consists of the production of a red color when an estrogen is heated with concentrated sulphuric acid or phenolsulphonic acid and the mixture subsequently diluted with water.

We found in the first place that the red color in the reaction mixture could be discharged by neutralizing the solution with sodium bicarbonate, and that restoration of the original red color could be achieved by reacidification, the color change occurring at a pH of somewhat less than 1.0. These facts suggested to us that the colored compound might be an oxonium salt. Now it is well known that certain $\alpha\ \beta$ diketones and o-quinones yield red colored oxonium salts in acid solution (cf. Kehrmann[26]). It appeared probable to us therefore that the red compound formed in the estrogen color reaction might be an oxonium salt of 3-hydroxy-16,17-diketoestratriene (XX), which, of course, under the conditions of the reaction would be partially sulphonated.



XX

We saw that if this explanation was the correct one, the following would be expected to occur:

(a) The red colored compound should be formed from estriol under much milder conditions than are necessary for its formation from estrone, since the $\alpha\ \beta$ dihydroxy-grouping of the former should yield an $\alpha\ \beta$ diketone more readily than would the $-CO-CH_2-$ grouping of estrone.

This was found actually to be the case. Heating estriol with 50 per cent sulphuric acid, or with lead peroxide in acetone solution and subsequently acidifying, or with perchloric acid, all yielded the typical red color. These treatments did not result in the formation of a red color from estrone. In passing I might mention that Mr. Darrach has recently standardized the conditions for the color reaction of estriol with perchloric acid, so that it is now possible to determine quantitatively the amount of estriol in an estriol-estrone mixture by a direct process.

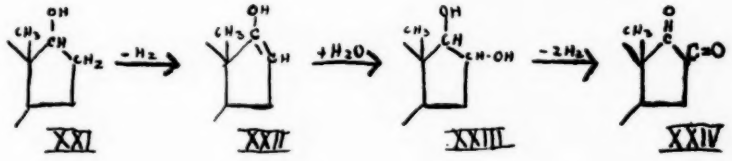
(b) The red color should be readily discharged by mild conditions of reduction, and the product should be partially sulphonated estriol and/or

a mixture of its C_{16} and C_{17} epimers, even though estrone was originally used in the reaction.

We again found that our anticipations proved to be correct. From the mixture obtained by discharging the red color in an estrone color reaction with zinc, tin, or sodium bisulphite, a product was obtained which behaved like estriol, and unlike estrone, in that it gave a red color on heating with 50 per cent sulphuric acid.

We were finally convinced that our explanation of the mechanism of the color reaction was essentially correct, when we found that the red colored solutions deposited a solid insoluble product on treatment with o-phenylenediamine, since the formation of quinoxalines on treatment with this reagent is characteristic of α β-diketones.

Shortly before we had arrived at this conclusion Pincus and Zahl[27] made the very important discovery that if estrone is administered to a pseudopregnant rabbit, or to an ovariectomized rabbit treated with progesterone, estriol appears in the urine. Experiments carried out on hysterectomized animals clearly showed that this *in vivo* hydration of estrone to estriol occurs in the uterus. Now, as has been seen, the main end-product of progesterone metabolism is pregnanediol, which is formed from the former by the addition of six hydrogen atoms. We therefore argued that if it was justifiable to assume from the work of Pincus and Zahl that the metabolism of endogenous estrogens in the pseudopregnant uterus is dependent upon the simultaneous conversion of progesterone to pregnanediol, then it might be logically expected to be a process involving the loss of six hydrogen atoms. That is to say we pictured endogenous estrogen being metabolized in the pseudopregnant uterus by a process involving three successive dehydrogenations in which progesterone was acting as a hydrogen acceptor. Now the work of Doisy and his co-



XXI XXII XXIII XXIV

workers[28] points to estradiol as the true endogenous ovarian estrogen. We therefore speculated on what might happen to estradiol (XXI) as a result of a three-stage dehydrogenation. It seemed likely that it might first lose

two hydrogen atoms to yield the enolic form of estrone (XXII), which might then add on the elements of water to form estriol (XXIII). The latter by two successive dehydrogenations might be expected to yield 3-hydroxy-16,17-diketoestratriene (XXIV)—the chromogen which we believe is formed in the Kober color reaction.

These entirely speculative arguments therefore led us to anticipate that hydroxydiketoestratriene might prove to be one of the main end products of simultaneous estrogen and progesterone metabolism. We therefore undertook a search for this substance in human pregnancy urine. We have not so far isolated it, and since isolation in a state of chemical purity is the only entirely satisfactory proof of the existence of a compound, we cannot be sure that our theories are correct. We have, however, obtained strong indications that human pregnancy urine does contain a chromogen which is not estrone or estriol and which displays many similarities in behavior to the chromogen formed in the Kober color reaction. We are hopeful, therefore, that our speculations concerning estrogen metabolism may be not far from the truth.

This theory of estrogen metabolism permits a new light to be thrown upon two interesting papers that have recently been published. Smith and Smith[29] have shown that the yield of physiologically active estrogenic material obtainable from human pregnancy urine can be greatly increased by the addition of zinc to the urine during the acid hydrolysis. We consider that this phenomenon may be due to the reduction of the diketone to estriol or its epimers, rather than to the reduction of estrone to estradiol as the authors themselves suggest. Patterson[30] has recently described a method for the chemical diagnosis of pregnancy based on the colorimetric determination of estriol by the Kober reaction. His process involves a preliminary treatment of the urine with sodium bisulphite, which he believes facilitates the performance of the color reaction by destroying various pigmented substances in the urine. We believe that the observed improvement in the color reaction after sodium bisulphite treatment, may be due to the reduction of the diketone originally present in the urine to estriol and its epimers.

In conclusion I must apologize for devoting so much of my time to speculative theories. Speculation is a poor substitute for experimental work, but in a new and rapidly expanding field such as this, it is perhaps justified since it often provides the stimulus that leads to the making of new laboratory discoveries.

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INFECTIOUS MONONUCLEOSIS*

JOHN R. PAUL

IT IS unnecessary to call attention to the fact that infectious mononucleosis is a disease which has been recognized for about fifty years under the term of glandular fever, but a disease which for the last fifteen years has been reclassified under a new name. Under the old name it was regarded more or less with indifference; under the new it has been rapidly accepted as a definite clinical entity, at least such has been the case in this country. Abroad, particularly in Germany, this has not been the case, and it is their loss, I should say. They still use in Germany the terms, "drüsenfieber" or "monozytenangina," both of which carry with them a nonspecific implication which cannot be dispelled by even the most exhaustive of their studies.[1,2]

It is also unnecessary, as well as impossible, to give a full account of infectious mononucleosis in an article of this type. Such descriptions may be found in some of the well-known articles describing this disease.[3,4,5] Nor will I attempt to discuss the various theories of its nature, nor the experiments which have been done in a search for an etiological agent. Instead, this article will be limited in its scope essentially to the clinical diagnosis of this disease.

It is of course of first importance to gain an adequate impression of the clinical picture. Next, one should have some knowledge of the two most important laboratory aids, the blood picture and the serological test. A knowledge of all three features should then make the diagnosis easy. In fact I think I am correct in saying that there are few diseases in which we are so fortunate as to have three such definite diagnostic aids, and yet, in spite of them, the disease probably escapes recognition very often. One has to be distinctly on the lookout for it. Often the clinical symptomatology is very mild; there seems to be such a thing as "walking infectious mononucleosis" and these and other cases may easily escape recognition if differential blood counts do not happen to be done. In fact, it is easy

* Delivered October 31, 1938, in the Eleventh Annual Graduate Fortnight.

for a great blind spot for the disease to develop. Under some circumstances this blind spot is so great as to almost blot the disease out of existence. This may have been the situation which universally prevailed with regard to glandular fever during the period of the World War. Professor Tidy[3] of London states that between the years of 1915 and 1920 he could not find a single reference to it in medical literature. Today the Index Medicus lists about twenty-five papers a year under the title of infectious mononucleosis. But in spite of local interest in some places, the distribution of the disease continues to be irregular. A student of ours who recently spent a year in postgraduate medical work in London, where much good work has been done on this disease,[3] tells me that the professor of medicine in one of the best known of their medical schools, informed him that he had never seen a case of infectious mononucleosis. But we may be subject to these same blind spots here. How did the Mt. Sinai Hospital of New York City happen to collect such a large series in a short space of time unless they were on the alert for them? Why does the disease seem to be so particularly prevalent in medical students? Is it possible that it is recognized only in those cases of upper respiratory infection in which consecutive blood counts are frequently performed?

Predisposing Features. I have already stated that I am not going to try to cover the whole clinical picture of the disease. Instead, I am going to draw largely upon experiences gained from our series of cases. This series is small. It comprises only fifty-one cases which have been studied at the New Haven Hospital, although additional information has also been obtained from about forty more cases seen elsewhere. In some respects our series gives a different picture from that mentioned in the usual textbook article.

Age. Most of the cases in our series occurred in young adult males who were between the ages of twenty and thirty. Rarely has the disease been seen on our Pediatric Service. This experience is not universal (Chart 1). There have been epidemics in which the cases were confined largely to children,[6] or school boys.[7] A remarkable one, in which the diagnosis was confirmed by serological tests, is that reported by Nolan[6] from the U. S. Naval Station at Coronado, California, in which 220 cases are mentioned as occurring among the juvenile members of sailors' families. Only five of these cases occurred in adults! The fact that it is a disease of young adults with us in New Haven, may be influenced by our university population, but I cannot believe that this is the sole deter-

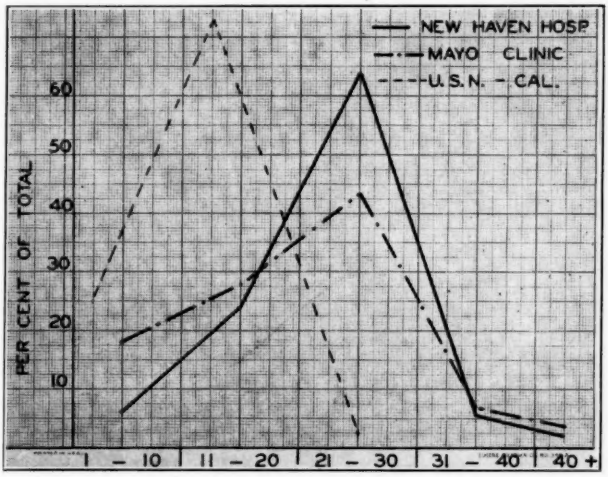

Chart 1—The age distribution of the New Haven Hospital series of 51 cases of infectious mononucleosis, compared with two other series: *i.e.*, 44 cases reported by Heck of the Mayo Clinic,[5] and a series of 220 epidemic cases of Nolan from the U. S. Naval Station in Southern California.[6] This last series suggests that there may be a special juvenile form of the disease, perhaps more prone to occur in epidemics than the adult form.

mining factor. Or, there may be a juvenile form of the disease which is prone to appear in epidemic form which we have not had the opportunity of seeing at our hospital.

Occupation. The use of the term infectious mononucleosis was first applied by Sprunt and Evans[8] to an acute illness of which six cases were described, four of which occurred in medical students. Since then there have been many comments on the extraordinary predilection which this disease has for students living in institutions, and particularly for medical students. Two-fifths of our cases occurred in individuals who were directly or indirectly associated with the New Haven Hospital (See Table 1).

But besides the "institutional" feature, whether medical or otherwise, the list of occupations in Table 1 indicates that we are dealing with a disease of those in sedentary employment. Is this because our patients happen to be individuals who are prone to be hospitalized for what

TABLE I

OCCUPATIONS OF 51 PATIENTS WITH INFECTIOUS MONONUCLEOSIS AT THE NEW HAVEN HOSPITAL

Hospital "Attendants"	*Others*
6 Medical students	8 Schoolboys or girls
5 Internes	8 University students
4 Nurses	3 Teachers
1 Technician	2 Bookkeepers
1 Social worker	2 Housewives
1 Orderly	2 Unspecified
1 Porter in hospital	2 Infants or children
1 Doctor's wife	1 Minister
	1 Architect
	1 Salesman
	1 Poolroom attendant
20	31

SEX Males 34 — Females 17

appears at first to be a trivial illness and they thus get the benefit of one or more blood counts? Certainly it is true that many cases are mild and the patient insists on remaining up and around, and probably this is more true than ever among the laboring class.

CLINICAL PICTURE

The clinical picture of infectious mononucleosis, as we know it, can be reviewed best here with the aid of a diagram illustrating clinical signs and laboratory tests exhibited by a more or less typical case (Chart 2). The diagram is helpful because the chronological order in which the signs appear is important. To begin with, it is hardly enough to speak of infectious mononucleosis as a disease characterized by general glandular enlargement, by mononucleosis and by an increased titer for sheep cell agglutinins, unless we appreciate that none of these signs may be present during the first week of the disease.

Incipient Symptoms. In our series of cases the onset has often been insidious and marked by nonspecific features such as sore throat, malaise, pain in the shoulders and neck, leukopenia, and an irregular fever. The temperature curve at this period is confusing. It may be similar to that

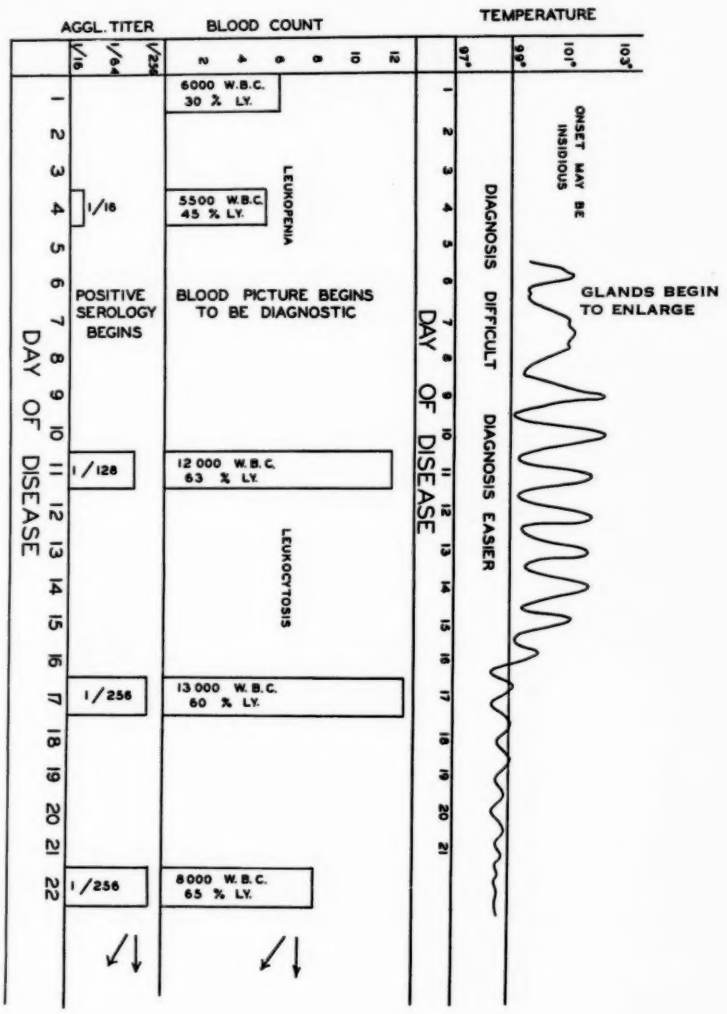

Chart 2—The course of a typical example of a moderately severe case of infectious mononucleosis, illustrated by the temperature curve, the blood picture, and the serological reactions.

TABLE II

SYMPTOMS AND SIGNS DURING THREE STAGES OF THE DISEASE AND THE PERCENTAGES IN WHICH THEY ARE RECORDED IN OUR SERIES

Prodromata or Early Symptoms (*Exclusive of fever and headache which is very common*)		*Important Signs and Symptoms of the 1st and 2nd Week of the Disease*		*Late and Convalescent Symptoms*
Sore throat	46%	Swollen glands	60%+	Glands may remain large for weeks
Malaise	38%	Red throat, Patches on tonsil, Stomatitis	60%	Severe cases may leave patients feeling below par for months.
Swollen glands	15%			
Chills	15%			
Cough	15%			
Eyes sore or pain behind eyes	12%	Petechiae on soft palate	10%	
Neck sore or stiff	12%	Eyelids swollen	10%	
Pain in shoulder	6%	Jaundice	10%	
Pain in abdomen	6%	Rash	10%	

seen in a mild case of typhoid fever. Chilly sensations are frequent at the onset and the fever is often up in the evening to 101° or 103° and down the following morning, so that for the first few days of the illness the patient may get up and go to his work as usual. This fact often confuses the physician, who, like the patient, may require several days to make up his mind that he is dealing with someone who is really sick. Thus, it is often not until the end of the first week that the diagnosis is established.

By the end of the first week the glands may be sufficiently enlarged to attract attention and the blood count also begins to become diagnostic. At about this time the sheep cell agglutinins may have risen to a titer of 1/32 or above, which is also diagnostic.

Signs and Symptoms of the Full-blown Disease. The classical physical signs of the disease are too well known to require attention here. That is, an acute illness of ten days to three weeks duration in which there is a gradual enlargement of lymph glands which does not go on to suppuration; enlargement of the spleen; a red throat and stomatitis often accompanied by patches on the tonsils and pharynx and with lesions with which Vincent's organisms are associated. The frequency with which some of these were found in our series is listed in Table II. Here the percentages with which these occurred are probably relative, because all of the items were not searched for diligently in every case. Three clinical

features which are not common but striking when they occur, will be mentioned briefly:

(a) *Ocular symptoms* probably occur more frequently than one would be led to believe. In their early stages they are characterized by pain in the eyes, or pain back of the eyes which regresses during the second week but leaves the patient with puffy eyelids. This may be a striking finding.

(b) A *papulo-macular rash* is an irregular symptom but one which has been emphasized, and properly so, by Tidy.[3] The eruption is diffuse or patchy and generally limited to the trunk. It is difficult to describe other than by the term morbilliform; in two of our cases it was classified by the attending physician as a "drug rash." It may be preceded, or accompanied by an enanthem consisting of petechiae on the soft palate.

(c) *Jaundice* is another manifestation present in the second or third week of the disease. It is often definite and striking. A recent report from Sweden emphasizes its importance.[9]

Convalescence. Infectious mononucleosis is a benign condition which is self-limiting in two or three weeks. However, it may be a long time (perhaps six months) before the glands return to normal size, and a long time before the patient feels like himself again. So prominent is this post-febrile depression that in severe cases it seems to be part of the disease. One of our patients ran a low grade fever for six weeks and was more or less prostrated during this whole period.

Blood Picture. Half of our cases showed an appreciable leukopenia during the first week of the disease. Here the counts ranged about 4,500 per c. mm., the lowest being 2,000 which occurred in two cases. This results from a granulocytopenia and during the first and second weeks one finds, besides this sharp reduction of polymorphonuclear neutrophiles, a decrease in filamentous forms giving rise to a so-called toxic blood picture. The disease may start, therefore, with an acute granulocytopenia and it may be worth considering what relationship this bears to the oral lesions, which, it will be recalled, are common in agranulocytosis of other types. During the first week the lymphoid cells may range from 28 to 60 per cent. It is also my impression (although I have not had occasion to confirm the degree to which this is true) that the characteristic abnormal lymphoid cells, so common in this disease, generally do not appear in diagnostic numbers at this time. In other words, one is fortunate if one can make a diagnosis from the blood picture during

the first week.

By the second week the total white count increases to give rise to a leukocytosis which may range from 8,000 to 20,000 leukocytes per c.mm. At this time lymphoid cells are found in from 40 to 85 per cent. This then is the stage of lympho-mononucleosis in which large and characteristic cells appear in considerable numbers in the blood. It is well known that some observers consider these cells to be absolutely diagnostic of the disease, others do not. Downey and McKinlay[10] gave in 1923 one of the best of the early descriptions of these lymphoid cells in infectious mononucleosis (or what they called acute lymphadenosis). In their article there is a colored plate drawn fifteen years ago but adequate for our purposes today. Here they describe three types of lymphoid cells distinguishable from the cells of acute lymphatic leukemia. Their differential point was, and still is, that the predominating large lymphoid cells of infectious mononucleosis are mature cells, in contrast to the immature cells of acute lymphatic leukemia.

Osgood[11] has later demonstrated that immature cells (lymphoblasts) may be found in infectious mononucleosis but that a more mature cell, for which he uses the term *prolymphocyte*, is the more typical cell. In size these are larger than polymorphonuclear leukocytes and this alone is enough to differentiate them from normal lymphocytes. Furthermore, the nucleus may have prominent nucleoli or intranuclear bodies and also fenestrations within the cell. Osgood believes these fenestrations to be another diagnostic sign in infectious mononucleosis.[12]

The characteristic blood picture with a leukocytosis ranging from 8,000 to 20,000 cells and the relative lymphocytosis with representatives of the lymphoid series above described, is probably a late (second and third week) manifestation of the disease. By the end of the third week the total leukocyte count drops, as do also the percentages of lymphoid cells. Leukopenia during convalescence lasting for some weeks or months, has been described.[3]

The red count is unaltered throughout the course of the disease, and this is another point helpful in eliminating lymphatic leukemia where anemia is so common.

Serologic Diagnosis

Reference has already been made to the presence of a unique and easily demonstrable antibody in this disease. Its discovery was the result

of an accidental finding and its real significance is still unexplained. Like the Wassermann test it is essentially a nonspecific reaction, but in syphilis we know the etiological agent, whereas in this disease we do not.

The story of this finding takes us back to the work of Forssman,[13] who in 1911 recognized the existence of certain principles with regard to the nonspecificity of certain antigen-antibody reactions. He demonstrated serological relationships between substances (now recognized as non-protein "haptens") in the cells of animals widely separated in the zoological system. The antibody in these reactions was, and has been described, therefore, as the Forssman antibody, but to define a Forssman antibody is difficult. Perhaps, as suggested by Landsteiner,[14] now that we know more about such antibodies and particularly their variations, the term "heterogenetic" or "heterophile" should be more properly applied to reactions of antibodies with antigens which are not related in their origin.

Only recently has the application of the Forssman principle found its way into clinical medicine. This occurred when Davidsohn[15] studied the "heterophile" response, in the form of lysins and agglutinins for sheep cells (erythrocytes), in the serum of patients who had been injected with horse serum. Horse serum contains a heterophile antigen and so it was natural that when it was injected into man, there should be a heterophile antibody response. Soon afterwards, during the course of a search for heterophile antibody responses in various clinical conditions, a high titer of such antibodies was discovered in the blood of patients with infectious mononucleosis.[16] Later Bunnell[17] proved that this finding was sufficiently unique to be of diagnostic value in this disease. The use of the test has increased the degree of confidence with which the disease can be diagnosed. Whether this is responsible for increasing the number of cases in our series is not known, but in any event from 1923 to 1930 we had only twelve cases, whereas in a similar length of time, from 1930 to 1936, we have had thirty cases.

It should be pointed out that normally, sheep cell agglutinins exist in low titer in the sera of most individuals. Seldom is this titer above $\frac{1}{8}$. In horse serum disease this titer may be enhanced up to 1/64 or higher. The normal antibody, which is easily demonstrable in the form of sheep cell agglutinins, and the serum disease antibody adhere to the type originally described by Forssman and originally known as the "Forssman antibody." In infectious mononucleosis the heterophile antibody exhibits

certain differences[18] which are sufficiently clear cut to differentiate it from this original "Forssman antibody." This difference can be readily brought out by absorption tests,[19,20] and these tests are not too complicated for the average clinical laboratory. At first thought it would hardly seem as if such a differential test would have much clinical value, for not often would serum disease and infectious mononucleosis be confused. But, as a matter of fact, it does have a clinical value and an important one. This is particularly true in the case of patients suspected of having infectious mononucleosis but in whom the titer is low (1/16 or 1/32). Here it is necessary to know whether one is dealing with what I shall refer to as the "I.M." antibody, or whether one is dealing with the antibody which occasionally exists in normal individuals. As early diagnosis is to be desired in this, as well as in other diseases, and as the titer of agglutinins is apt to be low (1/16 or 1/32) in the early stages of this disease, the clinical value of the differential (absorption) sheep cell test immediately becomes apparent.*

Appearance Time and Duration of Agglutinins. The general course of the serologic reactions in infectious mononucleosis is shown schematically in Chart 2. In the first week the titer is usually low (1/16). In the second and third weeks it averages 1/256 or slightly higher. Titers as high as 1/2000 were encountered twice in our series during this period of the disease.

Much work remains to be done with regard to the duration of the elevated sheep cell agglutinin titer. By and large, it is safe to say that it disappears fairly quickly; or, in other words, this antibody is apparently more transient than that seen in virus diseases or in a bacterial disease such as typhoid fever. Often the level had dropped to ⅛ by the fifth or sixth week in our series of cases of infectious mononucleosis. In one case, to which I have already referred, it remained at a level of 1/64 for seven weeks and the patient continued to have fever, mononucleosis and considerable malaise during this period. This patient eventually recovered but we believe that she represented an example of a chronic or subacute form of the disease. Exceptionally high titers may also require many weeks before returning towards normal, as in the case shown in Table III.

* Actual details of performing these serological tests will not be given in this article. For methods, there are many sources to which the reader may be referred.[20] It should be mentioned, however, that one of the main features of the test for the differentiation of the I.M. antibody from the normal sheep cell agglutinin, is that the I.M. antibody is but slightly or partially absorbed by suspensions of guinea pig kidney, whereas the normally occurring sheep cell agglutinin is wholly absorbed by this antigen. For a description of the methods of performing these tests the reader is referred to the work of Beer[19] and of Davidsohn.[20]

TABLE III

BLOOD COUNTS, SHEEP CELL AGGLUTINATIONS, KAHN AND WASSERMANN REACTIONS IN A CASE OF INFECTIOUS MONONUCLEOSIS

Date	*Day of Disease*	*W.B.C.*	*Lympho-cyte %*	*Sheep Cell Agglutinin Titer*	*Kahn*	*Wassermann Alc.*	*Wassermann Chol.*
1/10/38	13	6,050	49	1:512	++++	—	++++
1/15/38	18	4,500	21	1:4096	++++	—	++++
1/22/38	25			1:2048			
2/ 5/38	39			1:512	++		++++
2/11/38	45	7,050	28	1:512	+	—	—
2/19/38	53			1:512	+	—	++
3/ 5/38	70			1:256	—	—	—
4/ 5/38	101			1:256	—	—	—

A recent report from Japan[21] describes the results with convalescent sera which were tested from about ten patients. Blood was obtained from one to three months after the acute illness. Half of them still showed a titer of 1/16 or over. Two were still above 1/64.

Cases with Negative Serology. Another question is, how frequently does this reaction fail to occur in proven cases of infectious mononucleosis. In the New Haven series, one or more tests were performed upon thirty-seven patients and in only four of these did the titer prove to be less than 1/32. In other words, 90 per cent of our cases had positive serology. This may indicate that we are relying too much upon the sheep cell test for our diagnosis. In Nolan's[6] epidemic series, previously referred to, he mentions that all of those tested among his total of 220 cases had agglutinins of from 1/20 to 1/320.

On the other hand, Davidsohn[22] who has done extensive work on this subject has stated that it may be necessary to designate many patients by the term, *seronegative* cases of infectious mononucleosis. From the Mt. Sinai Hospital[23] comes the surprising statement that the heterophile antibody reaction is positive in only 40 per cent of the cases. Obviously, much more work is needed on this point, particularly as there is as yet little data as to what the differential (absorption) test would reveal in the cases with low titers and presumably negative tests.

Positive Wassermann or Kahn Reactions. Shortly before the heterophile test began to be used in this disease, a report from Germany described a temporarily positive Wassermann reaction in a patient suffering with what was called Monozytenangina.[24] This was in 1928. In 1930

Weber,[25] in England, called attention to the fact that in glandular fever temporarily positive serological tests for syphilis may occur. Several reports have subsequently followed; among the most recent is that of Bernstein[26] from the Johns Hopkins Hospital. In his series of thirty-seven cases a false-positive Wassermann reaction was noted in six.

Thirty-eight cases from the New Haven Hospital series have had serological tests for syphilis and of these, three yielded positive results either by the Kahn or Wassermann test. This is not a high percentage—that is, 8 per cent. It may be compared with the 16 per cent incidence of positives noted in the Johns Hopkins series. In both series, however, the routine tests for syphilis were probably made early in the disease. Had they been made upon samples of blood taken late in the disease, it is quite probable that the incidence of transiently positive reactions would have been higher. In fact, this is suggested by the Japanese observation,[21] noted earlier in this paper, in which samples of sera were obtained and tested from one to three months after the acute disease. About 40 per cent of these sera are listed as having a positive Wassermann!

When this does occur it is indeed striking, as is to be seen in one of our cases, reported by Sadusk[27] (See Table III). Here the Kahn, Wassermann and heterophile antibody tests returned towards normal more or less simultaneously. This again raises questions about the significance of the peculiar antibody or antibodies present in this disease. Surely no ordinary bacterium with which we are familiar, can cause such an immune response. Consequently, although the problem of etiology may be said to be still open in this disease it would at least seem, from immunological evidence, that an ordinary bacterium can perhaps be eliminated.

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IN MEMORIAM

Doctor George Edmund de Schweinitz

In the death of Doctor George Edmund de Schweinitz on August 22, 1938, Medicine lost one of its most distinguished men, and Ophthalmology its greatest leader. Doctor de Schweinitz, son of the Right Reverend Edmund Alexander de Schweinitz, Bishop in the Moravian Church, was born in Philadelphia on October 26, 1858, attended the Moravian Parochial School in Bethlehem, Pennsylvania, and later entered the Moravian College in 1872. In 1876 he received the degree of Bachelor of Arts and in 1878 the degree of Master of Arts. Three years later he graduated with honors from the School of Medicine of the University of Pennsylvania, winning the Hodge Gold Medal for "Proficiency in Anatomy". At that time he was also awarded the Henry C. Lea prize for his thesis on "Painful Tumors with Special Reference to Neuromata."

Immediately after leaving medical school Doctor de Schweinitz was appointed a resident physician to the University of Pennsylvania Hospital and later to the Children's Hospital, both in Philadelphia, and served with these institutions for a period of two years. He began practicing medicine in 1883 and devoted the next four years to general work. It was about this time that he began to devote his attention to diseases of the eye, especially the relation of the eye to general diseases. He developed this important part of ophthalmology by many contributions. In a very brief period he won the respect and confidence of the older men in the profession, who referred many unusual and difficult cases to him for study.

Doctor de Schweinitz later served as ophthalmic surgeon to the Children's Hospital, Philadelphia General Hospital, Orthopedic Hospital and Infirmary for Nervous Diseases. He was consulting ophthalmologist to the Chester County, Bryn Mawr, and Philadelphia Polyclinic Hospitals.

Besides winning distinction as an ophthalmological surgeon, Doctor de Schweinitz gained international fame as a teacher and lecturer. His first experience in teaching medicine began in 1883 as quiz master on therapeutics. For five years, he conducted the most popular and successful quiz in the university, and for three years was prosector to Doctor Joseph Leidy, professor of anatomy at the University of Pennsylvania, and the greatest American student of biology. From 1891 to 1892 he was a lecturer on medical ophthalmoscopy at the University of Pennsylvania and from 1891 to 1894 was professor of ophthalmology in the Philadelphia Polyclinic and College for Graduates in Medicine. In 1892, Doctor de Schweinitz was appointed clinical professor of ophthalmology in the Jefferson Medical College, and in 1896 professor of ophthalmology. He was professor of ophthalmology at the medical school of the University of Pennsylvania from 1902 to 1924 and at the Graduate School of Medicine from 1924 to 1928 when he became emeritus professor. Because of his unusual ability as an administrative officer he was appointed a member of the Board of Trustees of the University of Pennsylvania. In 1924 he served as honorary chairman of the Board of Medical Affairs.

During his long and successful career, and as a result of the great esteem in which he was held, Doctor de Schweinitz received numerous awards, including several honorary degrees. An honorary degree of Doctor of Laws was conferred by the University of Pennsylvania in 1914, and one of Doctor of Letters of Humanity by the Moravian College in 1922. The University of Michigan conferred an honorary degree of Doctor of Science in 1923 and Harvard University conferred a like degree in 1927. Among other awards was the Alvarenga Prize in 1896 of the College of Physicians of Philadelphia for his essay on "Toxic Amblyopias" to which subject he had devoted much of his time.

In 1926, the American Medical Association presented him with the Howe medal in

recognition of his scientific and scholastic attainments. In presenting the award, Doctor Walter Parker stated, "The Committee, by unanimous consent and with the approval of the Council, presents the Howe Medal to Doctor George E. de Schweinitz, a member of this Society, who has more than met every requirement of the deed of gift. As a teacher he has been an inspiration to his students and a guide and counselor to his fellow workers. His textbook is the outstanding treatise on ophthalmology in this country, and the list of subjects investigated by him includes not only problems of ophthalmology but those of general medicine, neurology, and pathology. No other member of our profession has had a wider range of interest."

In 1930, Doctor de Schweinitz was presented with the Leslie Dana Medal for outstanding work in behalf of the prevention of blindness. The medal bore the inscription of "wise, learned, patriotic, teacher and guide."

Doctor de Schweinitz was elected to membership in numerous societies in this country, and in many of these was honored with posts of distinction. He served as president of the American Ophthalmological Society in 1915. From 1910 to 1913 he was president of the College of Physicians in Philadelphia. He was one of the organizers of the Association for Research in Ophthalmology and was elected to honorary fellowship in 1934. One of the greatest honors conferred upon Doctor de Schweinitz was his election to the presidency of the American Medical Association in 1922. Doctor de Schweinitz was a member of the Section on Ophthalmology of the American Medical Association, American Ophthalmological Society, American Academy of Ophthalmology and Otolaryngology, National Society of Social Science, American Philosophical Society and was Honorary Fellow of The New York Academy of Medicine. In 1922, he was president of the International Congress of Ophthalmology in Washington. He became a life member of the American College of Surgeons and served on the Board of Governors from 1916 to 1919 and on the Board of Regents from 1919 to 1924. He was also director of the Philadelphia Library Company and vice-president of the Pennsylvania Institute for the Instruction of the Blind.

Doctor de Schweinitz was extremely interested in the broad international aspects of ophthalmology and spent a good deal of time abroad. He took active interest in the important English and French societies and was a member of the Ophthalmological Society of the United Kingdom, the Société française d'ophtalmologie and the Société d'ophtalmologie de Belgique. He was an honorary member of the Ophthalmological Section of the Royal Society of Medicine and of the Hungarian and Egyptian Ophthalmological Societies. The Bowman Lecture on "Ocular Aspects of Pituitary Body Disorders" which he gave in London in 1923, is one of his most outstanding works. In 1924, he discussed the "Ocular Manifestations of Focal Infections" at the annual meeting of the Société française d'ophtalmologie. At this meeting he was honored with the award of a bronze plaquette with the inscription "Quaerere et Praebere Lucem."

When the United States entered the World War, Doctor de Schweinitz was appointed lieutenant-colonel in the Medical Reserve Corps. As a result of the excellent service he rendered both in this country and abroad, he was appointed brigadier general in the Reserve Corps in 1922, and became a member of the editorial board of the Medical and Surgical History of the War.

During the many years Doctor de Schweinitz devoted to active practice as an ophthalmologist and to the teaching of ophthalmology, he made many notable contributions to ophthalmic literature. He wrote several hundred articles on ophthalmologic and neurologic subjects and did considerable research work. The one who had the greatest influence on his career was S. Weir Mitchell who gave him his lasting interest in the neurological aspects of ophthalmology. The greatest literary contribution made by Doctor de Schweinitz was his authoritative textbook on ophthalmology, "Textbook on Diseases of the Eye", which appeared in 1892. There have been ten editions and several reprintings of this valuable volume which is used in every country in the world. He also collaborated with Doctor B. Alexander Randall of Philadelphia in editing a "Textbook on Diseases of the Eye and Ear, Nose and Throat." He was the American editor of Haab's volumes on "Ophthalmoscopy", "Ex-

ternal Diseases of the Eye" and "Operative Ophthalmology." He and Doctor Holloway wrote on "Pulsating Exophthalmos," and edited with Doctor Jackson "The Ophthalmic Year Book" for a number of years.

One of Doctor de Schweinitz's most dominant characteristics was his willingness to assist younger men in their work. He was never too busy to advise young ophthalmologists, who affectionately called him "King George" because of his distinguished appearance and bearing. His generosity to younger men was shown by his eagerness to collaborate with them in writing articles. He enjoyed writing, and loved to help his assistants obtain recognition for their literary contributions. He had an exceptionally alert mind and had such a concise, clear view of so many things that it could not but be reflected in the writings of the younger men he taught. He was deeply interested in scientific research and gave encouragement, assistance and unstinted praise to those who achieved results. He had hosts of friends, both in and out of the medical profession and many benefited by his skill as surgeon and ophthalmologist.

In writing the history of ophthalmology in the twentieth century, the work of George Edmund de Schweinitz will stand out with that of other great men like Wilmer and Fuchs. It is certain that the inspiration from his work and character will continue to be a force driving ophthalmology to ever greater heights.

CONRAD BERENS

DEATHS OF FELLOWS

DENENHOLZ, AARON: 1904 Avenue I, Brooklyn, New York; born in Austria, June 16, 1875; died in Brooklyn, New York, December 1, 1938; graduated in medicine from the New York University Medical School in 1897; elected a Fellow of the Academy, December 3, 1908.

Dr. Denenholz had been at one time consulting gynecologist to the Manhattan State Hospital.

FORD, WILLIAM MILLER: 130 East 67 Street, New York City; born in Brooklyn, New York, November 30, 1878; died in New York City, November 26, 1938; graduated in medicine from the University of Virginia in 1899; elected a Fellow of the Academy Mar. 3, 1904.

Dr. Ford, who was a former president of the medical board of St. Vincent's Hospital, was attending surgeon to that institution from 1905-26 and director of gynecology from 1927-31. He was consulting gynecologist to the New York Hospital for Ruptured and Crippled since 1921 and clinical professor of obstetrics at New York University from 1920-33.

Dr. Ford was a Fellow of the American College of Surgeons, the American Medical Association and a member of the New York Obstetrical Society and the County and State Medical Societies.

LALLY, JORDAN: 30-60 29 Street, Long Island City, New York; born in Philadelphia, Pennsylvania, December 2, 1893; died in New York City, November 26, 1938; graduated in medicine from the University of Vermont in 1921; elected a Resident Fellow of the Academy April 2, 1931.

Dr. Lally was a member of the American Medical Association, the American Public Health Association and the County and State Medical Societies.

SITTENFIELD, MAURICE JOSEPH: 29 West 74 Street, New York City; born in Lubarsch, Germany, March 17, 1877; died in New York City December 1, 1938; graduated in medicine from Bellevue Hospital Medical College in 1898; elected a Fellow of the Academy November 7, 1912.

Dr. Sittenfield was at one time associate surgeon to the Mount Sinai Hospital. From 1909-34 he was assistant professor of pathology at the College of Physicians and Surgeons.

Dr. Sittenfield was a Fellow of the American Medical Association, a Fellow of the American College of Radiological and a member of the Radiology Society of North America, the American Radium Society, the American Association of Pathologists and Bacteriologists, the American Association for Cancer Research, the New York Pathological Society and the County and State Medical Societies.

Dr. Sittenfeld was the author of many papers and articles on radiology.